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BIOCHEMISTRY OF BACILLUS ACETOETHYLICUM WITH REFERENCE TO THE FORMATION OF ACETONE.*

By JOHN H. NORTHROP,† LAUREN H. ASHE,‡ AND JAMES K. SENIOR.‡,§

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 18, 1919.)

Several microorganisms[1] have been described as forming more or less acetone, and at least two[2] have been used on a commercial scale. Of these organisms, *Bacillus macerans* seemed the most desirable for the present work, inasmuch as it forms ethyl alcohol as a by-product. Since it was not possible to obtain a culture of the organism in this country, experiments were undertaken with the view of isolating the bacillus.

I. Isolation of Bacillus.

Samples of potatoes were obtained from various parts of the country. Cylinders were cut from these, put in test-tubes, and sterilized for 20 minutes in the Arnold. The tubes were then in-

* Published by permission of the Director of Chemical Warfare Service, U. S. A.

The present work was undertaken at the suggestion of the Council of National Defense in an attempt to develop a fermentation process for the production of acetone. Attention, therefore, has been centered on the acetone formation so that the work is incomplete in many other respects.

† Captain, Chemical Warfare Service.

‡ 1st. Lt., Chemical Warfare Service.

§ Lt. Senior collaborated in the early part of the work and developed the analytical methods used.

[1] Kayser, E., *Ann. Inst. Pasteur.*, 1894, viii, 737. Bréaudat, L., *Ann. Inst. Pasteur*, 1906, xx, 874. Schardinger, F., *Wien. klin. Woch.*, 1904, xvii, 207; *Centr. Bakteriol., 2te Abt.*, 1905, xiv, 772.

[2] F. Bayer and Co., *D.R.P.*, 283,107, July, 1913; 291,162, Jan., 1914; British Pat. No. 14,371, June, 1914. Delbrück, K., and Meisenburg, K., *U. S. Pat.* 1,169,321. Fernbach, A., and Strange, E. H., *U. S. Pat.* 1,044,368; 1,044,446; 1,044,447.

cubated. After several days one set of tubes containing potatoes from Berkshire County, Massachusetts, showed gas formation and crumbling somewhat as described by Schardinger.[3] The fermented potatoes from these tubes were distilled with a little water and the distillate was tested for acetone with paranitrophenylhydrazine. A strong positive test was obtained.

Microscopic examination of the culture showed the presence of large oval spores and many long thin rods. Some of the material was plated on 2 per cent glucose agar and gave small translucent colonies. Several of these were replated three times and then transferred to glucose agar slants. The culture was kept growing on this medium as it was found that the fermentative power remained constant under these conditions. Potato medium inoculated from these slants gave consistently positive tests for acetone. An organism was therefore at hand in pure culture which produced acetone from starch. The general cultural characteristics of the organism were studied and are summarized in Table I, in accordance with the chart of the Society of American Bacteriologists. The fermentation reactions and the experiments on the optimum conditions for the fermentation will be described in more detail below.

TABLE I.

Description of the Organism.

Described according to descriptive chart of the Society of American Bacteriologists.

Source. From old potatoes obtained from Berkshire Co., Mass., July 1, 1917.

Proposed name. *Bacillus acetoethylicum.*

I. **MORPHOLOGY.**

1. **Vegetative Cells.**

Motile.

From 24 hr. agar slant, 40° C.

Short rods 4 to 6$\mu\mu$ × 0.2 to 0.3$\mu\mu$.

No chains.

Ends rounded. Stain evenly with Loeffler's methylene blue or gentian violet. Gram-negative.

From 24 hr. 10 per cent corn media.

Same as above but occasional short chains.

From old (6 to 10 days) 10 per cent corn media.

Stain unevenly with deeply staining spot at end or in center.

[3] Schardinger, F., *Wien. klin. Woch.*, 1904, xvii, 207.

TABLE 1—*Continued.*

2. **Spores,** elliptical, form at end of rods. Stain easily with methylene blue or gentian violet. 0.5 to 1.0 $\mu\mu$ in diameter.

II. CULTURAL FEATURES.

1. **2 Per Cent Glucose Agar Slant.**
   24 hrs., 40°C. Moderate, spreading, effuse, dull, translucent, no odor.
   Shiny.
   Condensation water opaque.
2. **Potato.**
   24 hrs., 40°C. Gas bubbles all over medium, crumbles easily, no odor.
   2 to 3 days, 40°C. Medium sinks to greyish white paste
3. **Glucose Broth.**
   24 hrs., 40°C. Cloudy, no odor.
   2 to 3 days. Slimy mass in bottom.
4. **Litmus Milk.**
   24 hrs., 40°C. Bottom of tube white, no gas, odor, acid, or clot.
   36 hrs., 40°C. Milk red on top, rest white.
   72 hrs., 40°C. Same but coagulated; clot does not digest subsequently.
5. **Agar Plate Colonies.**
   2 per cent glucose agar, 24 hrs., 40°C.
   Growth slowly spreading.
   Round, outline irregular.
   Surface smooth.
   Elevation effuse.
   Edge entire or undulate.
   Internal structure coarsely granular.
6. **Sodium Chloride in Bouillon.**
   Inhibiting concentration 4 to 5 per cent.
7. **Nitrogen.**
   With sugar as carbohydrates obtained from peptone, proteins, or ammonium salts.
   With starch, same but cannot use ammonium salts.
8. **Best Medium for Long Continued Growth.**
   2 per cent corn in media with $CaCO_3$.

III. PHYSICAL AND BIOCHEMICAL FEATURES.

1. **Optimum Reaction of Media.**
   For growth, pH = 8.0 to 9.0.
   For fermentation, pH = 6.0 to 8.0.
2. **Vitality on Culture Media.**
   At least 6 months at room temperature.
   At least 1 month at 40°C.
3. **Temperature Relation.**
   Optimum temperature 40–43°C.
   Spores may be boiled at least 20 minutes.

TABLE 1—*Concluded.*

4. **Resistant to Drying.**
5. **Products of Reaction.**
   Formic acid.
   Ethyl, propyl, butyl alcohol.
   Acetone.
6. **Fermentation of Sugars, Etc.**
   Ferments the following sugars in 1 per cent solution with addition of $CaCO_3$ and peptone as nitrogen source, 15 cc. in test-tubes.
   Ferments: levulose and galactose under following conditions.[4]
   Medium: 1 gm. of KH_2PO_4, 1 gm. of $(NH_4)_2HPO_4$, 0.01 gm. of NaCl, 1.0 gm. of $CaCO_3$, 10.0 gm. of levulose, per liter. Put in tubes and sterilized as described by Schardinger; inoculated with pure culture of the bacteria and incubated 12 days at 40°C.
   Acetone = 8 to 9 per cent.
   Alcohol = 14 to 20 per cent.
   Starch does not ferment under these conditions.

Fermentation Reactions.

Temperature 37°. Time 10 days. Medium 2 per cent sugar, 0.5 per cent peptone, 2 per cent $CaCO_3$.

Substance.	Acetone.	Alcohol.	Substance.	Acetone.	Alcohol.
	per cent	*per cent*		*per cent*	*per cent*
Galactose	4–5	22–24	Dextrin	6–7	14–16
Maltose	6–7	23–24	Dextrose	9–10	22–23
Mannose	6–7	22–23	Levulose	8–10	24–25
Raffinose	8–10	22–23	Xylose	4–5	18–20
d-Arabinose	6–7	12–16	Glycerol	—	40–43
Ca lactate	—	—	Sucrose	8–9	24–26
Starch	8–10	20–24			

7. **Air Relation.**
   Facultative anaerobe.
8. **Slime Formation.**
   In 10 per cent sugar solutions, having a reaction of pH 8.0 to 9.0, large quantities of slime are formed so that the whole medium becomes very viscous. Under conditions of fermentation a small deposit of slime settles to the bottom.

IV. PATHOGENICITY.

1. **Non-Pathogenic to Mice.**

[4] Schardinger, F., *Centr. Bakteriol., 2te Abt.*, 1905, xiv, 772.

The organism resembles *Bacillus macerans* therefore in its cultural characteristics in so far as can be determined from the rather brief description given by Schardinger.[4] It differs from it, however, in that it ferments galactose and levulose under anaerobic conditions with ammonium salts as the source of nitrogen. This distinction seems sufficient to differentiate the organism from *Bacillus macerans*. The name *Bacillus acetoethylicum* is suggested as it indicates the formation of acetone and ethyl alcohol, the most striking characteristic of the organism.

II. Identification of the Products of Reaction.

Culture medium.—10 gm. of peptone, 100 gm. of potato starch, and 20 gm. of finely divided calcium carbonate were boiled with 2 liters of water in a large Florence flask. The flask was plugged with cotton and autoclaved for 3 hours. It was inoculated with the growth from one glucose agar slant of the organism and the cotton plug tightly covered with tinfoil to prevent evaporation.[5] The culture was then incubated at 40°C. for 14 days, made alkaline with sodium hydroxide, and distilled.

Acetone.—The presence of acetone was established by the formation of characteristic needles with paranitrophenylhydrazine, by the iodoform test, and by precipitation with mercuric sulfate.

Alcohol.—20 cc. of distillate were made up to 200 cc. and oxidized with potassium dichromate as follows.

50 cc. of diluted distillate, 100 cc. of 1:1 sulfuric acid (100 cc. of acid made up to 200 cc. with water), and 75 cc. of 2 N $K_2Cr_2O_7$ were put in a 500 cc. pressure bottle. Three other similar bottles were prepared. They were heated for 15 minutes on the boiling water bath and the contents of the four bottles were combined, cooled, and made alkaline with NaOH. 300 cc. were then distilled off, and the residue made acid with sulfuric acid and slowly distilled until the distillate was only faintly acid. The distillate was neutralized to litmus with NaOH and evaporated to small volume on the steam bath. It was then acidified with sulfuric acid and extracted five times with an equal volume of ether, and the ether extract combined and filtered. The filtrate was made alkaline with ammonia and allowed to evaporate at room temperature. A concentrated solution

[5] This is necessary since it was found that if the flask was merely plugged with cotton only traces of acetone would be found after fermentation. That this is due to evaporation is shown by the fact that if the escaping gas from the flask is allowed to bubble through water a positive test for acetone is obtained from the latter.

of silver nitrate was added slowly and the resultant precipitate collected in three portions, dried, and analyzed for silver.

The results are shown in Table II.

The last fraction was therefore probably nearly pure silver acetate, while the first two fractions must have contained more or less of the higher salts.

Acid.—The residue from the above distillation was made acid with phosphoric acid and steam-distilled until 1 liter of distillate had collected. This was made alkaline with ammonia and evaporated to 100 cc. It was made slightly acid with sulfuric acid and extracted four times with an equal volume of ether.

Ether Extract.—The ethereal extract was made slightly alkaline with ammonia and allowed to evaporate at room temperature. The residue gave

TABLE II.

Analysis of Silver Salts.

Fraction No.	Weight of salt.	Weight of silver.	Per cent of silver.
	gm.	*gm.*	*per cent*
1	0.1106	0.0663	59.95
2	0.1012	0.0635	62.87
3	0.1023	0.0658	64.32

Calculated for:	Silver acetate	64.64
	" propionate	59.64
	" butyrate	55.35

no precipitate with silver nitrate, showing the absence of any appreciable quantity of the higher acids.

Water Solution.—The water solution after extraction with ether was made alkaline with ammonia and evaporated to small volume. The addition of silver nitrate caused the formation of a silver mirror and a precipitate of metallic silver. The principal acid formed is therefore formic.

Acetone, ethyl alcohol, and formic acid are therefore the principal non-gaseous products of fermentation.

III. Effect of Varying Conditions on the Yield of Acetone.

The course of any fermentation may be considered as a function of the following variables: physiological state of the culture, temperature, air supply, reaction of the media, and the chemical and physical properties of the substrate. The effect of varying these

factors on the yield of acetone was studied with a view of determining the optimum conditions for its formation.

In order to carry out these experiments it was first necessary to have a convenient quantitative method for the determination of acetone and alcohol.

Determination of Acetone.—The method used was a modification of the Messinger-Huppert[6] method and was carried out as follows. An aliquot portion of the fermented media, containing 50 to 150 mg. of acetone, was diluted to 120 cc. with water, made alkaline with NaOH, and 40 cc. were distilled over into a 100 cc. volumetric flask. 40 cc. of water were placed in this flask before beginning the distillation and the delivery tube from the condenser allowed to project below the level of the water—this is necessary in order to prevent loss by evaporation. After 40 cc. had been collected, the distillate was made up to 100 cc. and 10 cc. were pipetted into a glass-stoppered bottle, 10 cc. of 10 per cent NaOH added, and an excess of 0.106 N iodine in KI was run in with constant agitation. The bottle was stoppered and allowed to stand 2 minutes. It was then made acid by the addition of 2 cc. of 1:1 sulfuric acid and the excess iodine titrated back with thiosulfate. This back titration should approximately equal the amount of iodine actually used in the determination.

The iodine solution was standardized against a solution of acetone made up by weight and containing twice the amount of alcohol, since it was found that this is approximately the ratio in which the two substances are formed. The correction for alcohol (which is 0.02 to 0.03 cc. per mg. of alcohol) is thus made automatically in the standardization. It was found under these conditions that 1 cc. of 0.1065 N iodine was equivalent to 1 mg. of acetone.

The accuracy of the method was checked by making control determinations with Van Slyke's[7] gravimetric mercury sulfate method. The agreement was always within 2 per cent which was more than sufficient for the purposes of this investigation.

Alcohol Determination.[8]—10 cc. of distillate containing less than 20 mg. of alcohol were pipetted into a magnesia bottle containing 15 cc. of 0.2 N potassium dichromate, 20 cc. of 1:1 sulfuric acid were added, and the stopper was clamped in. The bottle was then warmed and finally heated in the boiling water bath for 45 minutes. After cooling it was opened, the solution diluted to 150 cc., 10 cc. of 10 per cent KI were added, and the liberated iodine was titrated with thiosulfate, using starch as an indicator. A slight correction (0.03 cc. of 0.1 N iodine per mg.) is neces-

[6] Messinger, J., *Ber. chem. Ges.*, 1888, xxi, 3,366. Rakshit, J. N., *Analyst*, 1916, xli, 245.

[7] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxxii, 455.

[8] Dupré, *J. Chem. Soc.*, xx, 495.

sary for the acetone present and a correction of 0.15 to 0.30 cc. for the reagents. The solutions were standardized against solutions of alcohol made up by weight.

It was now possible to follow the changes in the production of acetone and alcohol.

Temperature.—In order to determine the optimum temperature for fermentation a series of cultures was made up and placed in incubators set at 36, 43, and 46°C. respectively.

Since the effect of the reaction, etc., was as yet undetermined, the cultures were made in what were considered the best conditions.

TABLE III.

Effect of Temperature.

Time of fermentation 15 days.

Medium: 2 per cent potato flour, 1 per cent peptone, 2 per cent $CaCO_3$.

Experiment No.	Acetone, in per cent of weight of starch.		
	36°C.	43°C.	46°C.
	per cent	*per cent*	*per cent*
1	5.7	8.4	7.7
2	7.1	8.8	7.0
3	6.5	8.6	7.5

Medium.—20 gm. of potato flour were mixed with a little cold water and poured with stirring into 700 to 800 cc. of boiling water containing 10 gm. of peptone. The suspension was boiled, 20 gm. of precipitated calcium carbonate were added, and the solution was made up to 1 liter. 50 cc. were measured into 120 cc. Erlenmeyer flasks which were plugged with cotton and sterilized 1 hour in the autoclave. They were then cooled and inoculated from a 24 hour glucose agar slant of the organism. The cotton plugs were tightly covered with tin-foil to prevent evaporation. In this and in all the other experiments the cultures were examined microscopically after incubation and any showing contaminating organisms were discarded. It was found that most of the common contaminating organisms destroy the acetone formed.

The results of the experiment are shown in Table III. It will be seen that the optimum temperature is about 43°C. and also that this is not due merely to the rate of fermentation since the cultures were left in the incubator 5 or 6 days after all signs of fermentation had ceased.

IV. Influence of the Reaction of the Media.

The study of the reaction of the media on the fermentation is complicated by the fact that, in order to maintain the reaction even approximately constant, it is necessary to use relatively high concentrations of buffer substances which may have a secondary effect on the fermentation. In order to reduce the amount of acid formed, a low concentration of carbohydrate was used. It is also necessary to note that the concentration of buffer is higher in the more alkaline cultures.

Cultures 1 to 10.—20 gm. of soluble starch, 5 gm. of peptone, and 34 gm. of Na_2HPO_4 were dissolved in 2 liters of water. 100 cc. of this solution were then pipetted into a series of 150 cc. Erlenmeyer flasks and titrated to the desired reaction with 0.25 M NaOH. After sterilization the reaction was measured colorimetrically, using Sörensen's standard solutions with bromocresol purple and phenol red.[9]

Cultures 11 and 12.—These cultures contained an excess of solid $MgCO_3$ in place of the phosphate. After sterilization the reaction was strongly alkaline to phenolphthalein and remained so throughout the fermentation.

The results are tabulated in Table IV. The optimum reaction for the formation of acetone under these conditions is therefore between pH 6.0 and 7.0. It was found that precipitated $CaCO_3$ would maintain the reaction at about this point and it was therefore used in the later fermentations.

It is interesting to note that in Cultures 11 and 12, which had a decidedly alkaline reaction, there was a very heavy growth of the organism but very little acetone formed. The optimum reaction for growth must therefore be on the alkaline side of the optimum for the production of acetone. Attempts were made to decide this question quantitatively by plating the cultures and counting the number of organisms. The results were very unsatisfactory, however, due to the fact that the organism collects in slimy masses which prevent accurate dilution. It was found, however, that no growth could be obtained on agar titrated to pH 6.4, although this is approximately the optimum for the production of acetone. These results seem to justify the conclusion that there are different optimum reactions for the different activities of the organism and possibly of bacteria in general.

[9] Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1.

It was found later while working with cane molasses, which is rather unfavorable as a culture medium, that no growth could be obtained if the reaction, to begin with, was more acid than pH 6.8, while a vigorous fermentation took place if the reaction was first adjusted to pH 8 to 9. This is due to the fact that the organism does not grow in the molasses except under the most

TABLE IV.

Effect of Reaction.

Temperature 42°C. Time of fermentation 11 days.

Media: Cultures 1–10, 1 per cent soluble starch, 0.25 per cent peptone, 1.7 per cent Na_2HPO_4.

Cultures 11–12, 1 per cent soluble starch, 0.25 per cent peptone, excess solid $MgCO_3$.

Titrated to reaction noted with 0.25 M NaOH.

	Culture No.											
	1	2	3	4	5	6	7	8	9	10	11	12
Reaction of media after sterilization, *pH*	5.0	6.0	6.0	6.4	6.4	6.8	6.8	7.2	7.2	7.6	10	10
Reaction of media after fermentation, *pH*	5.0	5.6	5.8	6.0	6.2	5.8	6.1	6.4	6.6	6.6	10	10
Acetone, *per cent*	—	8.9	8.6	9.2	10.0	6.6	6.8	6.4	4.4	6.6	Trace.	
Alcohol, *per cent*	No growth.	22.0	22.0	23.6	23.6			19.0		21.0	Heavy growth, very viscous.	

favorable conditions and hence when the reaction is too acid no growth takes place. When the media is more alkaline, however (pH 8 to 9), and therefore near the optimum for growth, the organism multiplies rapidly and at the same time produces sufficient acid to change the reaction to pH 6.0 to 7.0, where it is maintained by the carbonate present. This is about the optimum for acetone production. Table V shows the results of such a series.

It will be seen that the reaction of the culture which had been corrected to pH 9.0 to 9.5 was changed by the acid produced to pH 6.6 to 6.8 in the course of the first 24 hours.

TABLE V.

Effect of Reaction of Medium.

Temperature 42°C.

Medium: 5 cc. of cane molasses, 100 cc. of water, 2 gm. of $CaCO_3$, titrated with NaOH to reaction noted.

Time after inoculation.	Culture No.			
	1	2	3	4
	pH of media.			
days				
0	6.6	6.8	9.5	9.3
1	6.6	6.8	7.0	6.8
6	6.6	6.7	6.6	6.4
	Per cent of sugar as acetone.			
6	No fermentation.		8.5	9.0

V. Physiological Condition of the Culture.

It is well known that the character of the fermentation caused by some microorganisms may be decidedly changed according to the age and condition of the culture used for inoculation. In order to see if there was any such effect with *Bacillus acetoethylicum* a series of cultures was inoculated from the same parent culture at different times.

The data for this experiment are given in Table VI. It is evident that there is no marked effect upon the yield of acetone due to the age of the inoculating culture. It is necessary to note that the parent cultures were not tightly stoppered and that the volatile products of reaction therefore evaporated. This is shown by the fact that only 2 to 3 per cent of acetone was found in the parent flasks.

A second series of cultures was now made in which the flasks were tightly covered with tin-foil to prevent evaporation.

TABLE VI.

Effect of Age of Culture.

Media: Parent culture, 1,000 cc. of H_2O, 50 gm. of corn-meal, 20 gm. of $CaCO_3$, pH 7.6. Temperature 42°C.

Subculture, 50 cc. of H_2O, 5 gm. of corn-meal, 1 gm. of $CaCO_3$, 0.25 gm. of peptone. Incubation 11 days. Temperature 43°C.

Time after which subculture was inoculated.	Per cent of corn as acetone in subculture.		Microscopic appearance of parent culture.
	A	B	
days	*per cent*	*per cent*	
1	7.8	7.6	Rods.
2	7.9	7.1	"
3	8.0	8.4	"
4	8.0	8.5	" Few spores.
5	8.4	8.0	Mostly spores.
7	7.7	8.3	" "
9	7.8	6.4	" "
16	7.8	8.9	All spores.
30	7.15	6.8	" "
150	7.9	7.4	" "
Parent culture analyzed after 14 days..	2.8	2.4	

TABLE VII.

Effect of Successive Inoculation from Fermented Cultures.

Medium: 2.5 gm. of corn-meal, 0.5 gm. of peptone, 2 gm. of $CaCO_3$, 50 cc. of water.

Temperature 42°C.

Series.	Time of fermentation.	Acetone in flask No.				
		1	2	3	4	5
	days	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
A.	7	8.0	8.5	8.1	9.0	9.3
B, inoculated after 7 days from corresponding number in Series A.	7	5.1	5.3	3.8	4.0	3.4
C, inoculated after 7 days from corresponding number in Series B.	7	3.3	3.8	2.5	3.8	2.1

2.5 gm. of corn-meal, 0.5 gm. of peptone, 2 gm. of $CaCO_3$, and 50 cc. of water were sterilized in 120 cc. Erlenmeyer flasks. The flasks were inoculated and tightly covered with tin-foil. They were incubated 8 days. A second series of cultures prepared in the same way was then inoculated from these and the remainder of the solution analyzed.

The results are tabulated in Table VII. The yield of acetone is lower in each succeeding generation showing that the fermentative power of the organism is injured under these conditions. Since this was not the case in the preceding experiments it seems probable that the injury is due to some volatile product of fermentation. If the fermentation is carried out in closed vessels, therefore, it is not possible to use a culture which has undergone complete fermentation for the inoculation of fresh media. It will be shown in the experiments on a semicontinuous fermentation, however, that this may be done under certain conditions; *i.e.*, a shorter time of fermentation.

VI. Rate of Formation of Acetone and Alcohol.

In the preceding experiments the cultures were left in the incubator until all signs of fermentation had ceased. In order to determine the rate of formation of the products three cultures were inoculated and analyzed at intervals.

TABLE VIII.

Rate of Formation of Acetone and Alcohol.

Medium: 10 gm. of potato starch, 4 gm. of peptone, 10 gm. of $CaCO_3$, 500 cc. of water. Temperature 42°C.

Time analyzed as noted.

Time after inoculation.	Culture No.						Average.		Ratio alcohol to acetone.	
	1		2		3					
	Acetone.	Alcohol.	Acetone.	Alcohol.	Acetone.	Alcohol.	Acetone.	Alcohol.	By weight.	By mols.
days	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
3	4.6	12.3	4.0	11.4	3.3	10.7	3.9	11.5	2.9	3.65
4	7.4	16.1	5.6	13.5	4.6	12.3	5.9	13.9	2.3	2.9
5	8.9	18.1	7.4	16.6	6.1	14.7	7.5	16.5	2.2	2.8
6	9.2	18.9	8.6	18.4	7.4	18.0	8.4	18.4	2.2	2.8
7	9.3	19.4	9.0	19.4	7.7	18.1	8.7	18.9	2.2	2.8
9	9.3	19.5	9.1	19.4	8.3	20.0	8.9	19.6	2.2	2.8
13	9.4	19.5	9.0	19.3	8.8	20.2	8.7	19.6	2.2	2.8

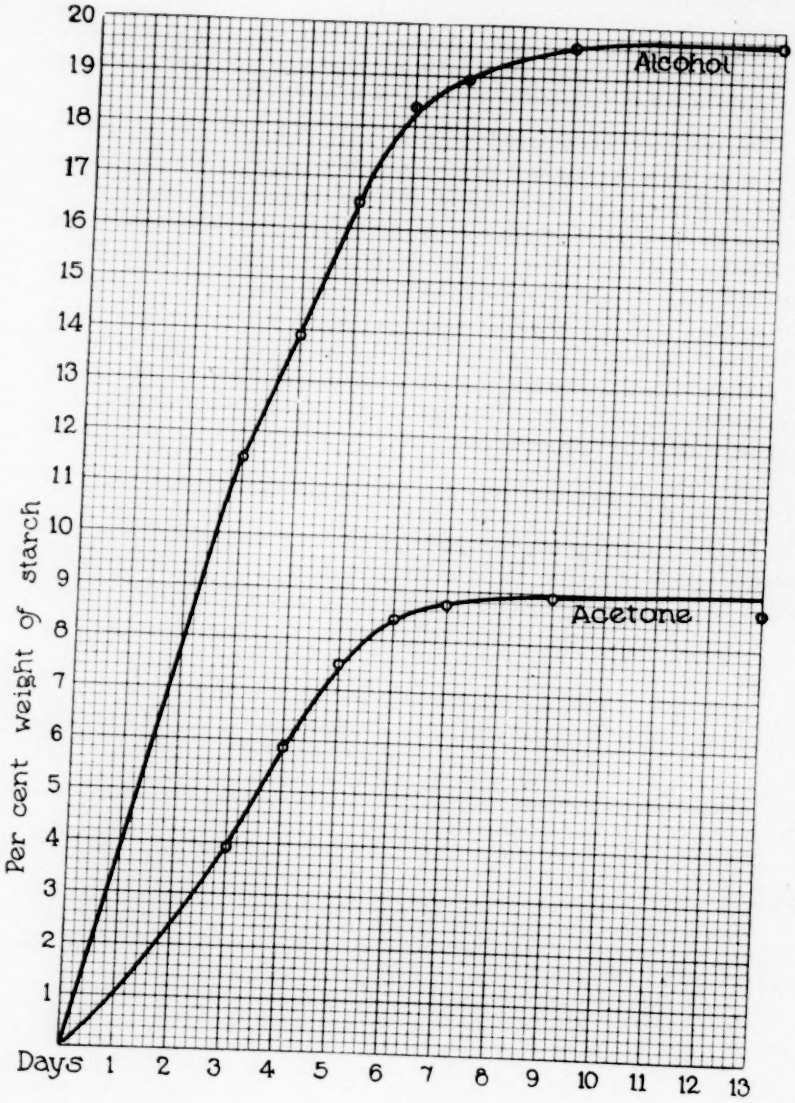
Alcohol
Acetone
Per cent weight of starch
Days
1
2
3
4
5
6
7
8
9
10
11
12
13
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1

Fig. 1.

500 cc. of water, 10 gm. of potato starch, 4 gm. of peptone, and 10 gm. of $CaCO_3$ were boiled in a 1 liter Pasteur flask. The flask was autoclaved 1 hour, inoculated, and incubated at 42°. Two similar flasks were prepared. Samples were withdrawn and analyzed for acetone as shown in Table VIII and in Fig. 1. Unfortunately the analyses were not made until the third day, so that the first part of the curve is missing. It will be seen, however, that the ratio of alcohol to acetone decreases from 2.9 to 2.2 (by weight) where it remains constant. The molecular ratios were calculated on the assumption that the alcohol formed is all ethyl alcohol—this is not strictly true.

TABLE IX.

Effect of Size of Inoculation.

Medium: 5 gm. of corn-meal, 2 gm. of $CaCO_3$, 0.5 gm. of peptone, 50 cc. of H_2O in 120 cc. flasks.

Temperature 42°C.

Inoculation.	Per cent of corn as acetone.					
	Flask No.	After 5 days.	Flask No.	After 7 days.	Flask No.	After 10 days.
		per cent		*per cent*		*per cent*
1 slant each.	1	6.4	4	7.1	7	8.5
	2	6.4	5	7.6	8	8.7
	3	6.2	6	8.0	9	8.1
0.1 " "	10	7.0	13	7.4	16	7.9
	11	7.0	14	7.5	17	8.0
	12	6.0	15	7.4	18	9.4
0.01 " "	19	6.4	22	7.1	25	8.9
	20	6.4	23	7.4	26	8.3
	21	6.9	24	7.4	27	8.2
0.001 " "	28	6.5	31	7.3	34	8.2
	29	5.6	32	6.8	35	8.1
	30	5.6	33	6.2	36	7.8

Under the conditions of the preceding experiment the fermentation requires 7 to 9 days. It seemed possible that this time might be shortened by using a larger inoculation. This was tested as follows.

A series of flasks was prepared containing 5 gm. of corn-meal, 2 gm. of $CaCO_3$, 0.5 gm. of peptone, and 50 cc. of water. They were sterilized, inoculated with varying amounts of the organism, incubated, and analyzed as usual.

Inoculation.—The growth from 10 agar slants was washed off in 10 cc. of sterile salt solution. Flasks 1 to 9 were then inoculated with 1 cc. of this suspension, equivalent to the growth from one slant; Flasks 10 to 18 with 1 cc. of a 1: 10 dilution, etc.

The results of the series are shown in Table IX. The size of the inoculation, within the limits of this experiment, evidently has very little effect on the time required for the completion of the fermentation. It was noted that fermentation commenced a little sooner with the heavier inoculation but since about the same time was required to complete the fermentation little is gained.

VII. Fermentation of Various Sugars.

The optimum conditions for the fermentation being known, it was now possible to determine the yields produced by the various sugars. The results of these experiments have been given in Table I. The organism ferments pentoses as well as hexoses and starch. No acetone is formed from glycerol which is transformed very largely into alcohol.

VIII. Experiments on Corn, Molasses, Etc.

The cheapest sources of starch or sugar are corn, molasses, and potatoes. A series of fermentations was therefore run to determine which of these substances would furnish the best yield.

The results of this series of experiments are summarized in Table X. It appears from these results that corn is the most favorable material for fermentation commercially. Experiments were therefore undertaken to see if the yield could be increased.

The influence of the following factors was studied: (1) addition of nitrogenous material; (2) effect of varying the air supply; and (3) concentration.

The results of the experiments on the addition of nitrogenous material and on the effect of the air supply are summarized in Table XI. The addition of nitrogenous material as peptone or yeast evidently increases the acetone yield considerably when little air is admitted to the culture. Aerobic conditions cause the same increase in the yield of acetone with, however, a decrease in the yield of alcohol. Addition of nitrogen under these condi-

tions causes no further increase in the amount of acetone produced. This indicates either, first, that the organism requires less nitrogen under aerobic conditions, or second, that it is able to use some of the nitrogenous material present in the corn which it cannot use in the absence of air. The latter explanation is probably correct.

TABLE X.

Yield of Acetone from Various Raw Materials.

Temperature 42°C. Time of fermentation 10 days.

	Substrate.			
	Corn (per cent of dry weight).	Potatoes (per cent of fresh weight).	Cane molasses (per cent of weight of total sugar content).	Beet molasses (per cent of weight of total sugar content).
Acetone	6.1	2.6	9.0	9.5
"	6.0	2.5	10.1	10.5
Alcohol	13.5	6.0	24.7	26.1

TABLE XI.

Effect of Air and Nitrogen on Yield from Corn.

Temperature 42°C. Time of fermentation 8 days.

Medium: 2 gm. of corn-meal, 50 cc. of water, 2 gm. of $CaCO_3$ in different size flasks and with peptone added as noted.

	Size of flasks.					
	120 cc.	120 cc.	120 cc.	500 cc.	500 cc.	500 cc.
Peptone added, *gm.*	0	0.5	1.0	0	1.0	1.0
Acetone yield (per cent of corn)	5.9	9.4	9.0			
" " " " " "	5.9	8.1	8.3	10.5	11.0	10.1
" " " " " "	6.1	9.5	9.6	11.5	10.0	9.8
Alcohol yield (per cent of corn)	15.0	20.1	21.0	15.6	17.9	22.0
" " " " " "				17.8	18.0	21.2

It will be noted that the corn yields about the same per cent of acetone as does corn-starch; *i.e.*, it ferments as though it were all starch whereas it contains not over 60 per cent. There is evidently some condition changed which causes the organism to produce a higher yield of acetone from starch present in the corn than it does from purified corn-starch. It seemed possible that this might be due to the presence of inert material. Many sub-

stances, such as cotton, asbestos, filter paper, etc., were tried but with no noticeable result. The addition of corn protein to the purified corn-starch was also without effect.

Concentration.—The results of a series of cultures of increasing concentration are summarized in Table XII. The maximum concentration which will completely ferment is about 8 parts of corn to 100 of water.

Experiments with Molasses.—The results in Table X show that both cane and beet molasses can be fermented with fairly good yields. Several experiments on the addition of peptone, phosphates, etc., were made, without however causing any increase in the yield.

TABLE XII.

Concentration of Corn.

Temperature 42°. Time of fermentation 12 days.
Medium: 50 cc. of water, 2 gm. of $CaCO_3$, 1 gm. of peptone in 120 cc. Erlenmeyer flasks with amount of corn-meal as noted.

Acetone (in per cent of corn).				
2.5 gm. of corn-meal.	3.25 gm. of corn-meal.	5.0 gm. of corn-meal.	7.5 gm. of corn-meal.	10.0 gm. of corn-meal.
9.0	9.0	8.3	5.6	5.0
9.3	8.9	7.7	5.4	5.0

Cane molasses is usually neutral or slightly acid and requires the addition of small amounts of alkali in order to bring it to the proper reaction.

The main disadvantages from a manufacturing standpoint of the fermentation as carried out in the preceding experiments is the long time required for fermentation and the difficulty of keeping the culture sterile.

It was noted in the cultures which had undergone fermentation that the calcium carbonate was held together by a thick viscous slime. Smears made from this material showed the presence of large numbers of the organism whereas smears made from the supernatant liquid showed comparatively few. It seemed probable, therefore, that if some inert material could be added to the culture so as to retain this slime and allow the clear liquid to drain off, most of the organisms would be retained and could be used to start the succeeding fermentation. Experiments showed

that this could be done. The fermentation was now semicontinuous and considerably more rapid. Owing to the clogging up of the cultures when corn was used these experiments were carried out with molasses. The cultures were grown in 500 cc. Florence flasks with a small piece of tubing fused to one side as in a Pasteur flask. The mouth of the flask was plugged with cotton and covered with tin-foil and the side arm was closed by a piece of rubber tubing and a glass plug. The flasks were ma-

TABLE XIII.

Semicontinuous Fermentation.

Temperature 42°C. Time of fermentation 48 hrs.

Medium: 500 cc. Florence flasks with side necks, filled with broken twigs. 6.5 per cent beet molasses (by volume) containing 70 mg. of total sugar per cc. pH = 8.0 to 9.0. Inoculation, 1 slant each. Emptied and refilled every 48 hours.

Fermentation No.	Per cent of sugar as acetone in culture No.			
	1	2	3	4 (no twigs).
	per cent	*per cent*	*per cent*	*per cent*
1	3.3	2.15	3.1	3.0
2	8.1	3.75	4.5	4.2
3	7.8	10.2	6.5	2.6
4	7.0	7.5	8.1	2.0
5	7.7	8.1	9.4	1.1
6	9.6	9.3	8.2	Trace.
7	7.2	9.6	8.1	
8	9.0	9.0	8.2	
9	6.8	8.4	8.7	
10	8.4	8.5	7.9	
11	8.1		9.2	
12	8.0			
13	8.2			

nipulated for refilling, etc., in the same way as an ordinary Pasteur flask. Almost any inert material such as corn cobs, coke, branches, etc., could be used to fill the flasks. In the experiments tabulated in Table XIII, branches were used. The flasks were filled with broken twigs, diluted molasses poured in, and the flasks plugged, sterilized, and inoculated as usual. At the times stated in the table the fermented medium was poured out and fresh sterile medium put in from a similar flask.

It will be seen that, under these conditions, the fermentation is complete in 48 hours and that the residue from an old fermentation is able to start a new one. The medium does not become sufficiently acid to stop the fermentation even though no neutralizing material is present. This is due partly to the fact that the fresh medium is made alkaline (pH 8 to 9). This, however, is not the complete explanation since the same medium when inoculated fresh from an agar slant develops acid and ceases to ferment. Controls without any inert material fermented slowly and soon stopped producing acetone. On the other hand, fresh cultures inoculated with a large volume (20 per cent) of the old fermented medium ferment slowly and usually show contaminating organisms. Smears made from the continuous cultures occasionally showed contaminating organisms also. These contaminations, however, did not affect the production of acetone as a rule, and often disappeared after one or two refillings.

In these experiments each succeeding fermentation is inoculated from the preceding one without any lowering of the yield. In the experiments described in Table VII it was found that this procedure resulted in lower and lower yields. The difference is due to the fact that, in the semicontinuous cultures just described the fermentation is complete in 40 to 50 hours whereas in the first series 7 days are required. This explanation is borne out by the fact that, if the cultures are refilled every 5 or 6 days instead of every 48 hours, the yields steadily decrease in the same way as was found in the early experiments.

These results show that the time of fermentation can be very materially shortened under these conditions and that the danger of contamination is also lessened. Attempts were made to make the process more nearly continuous by adding the fresh mash to the bottom of the fermenting culture and allowing the fermented mash to flow off at the top. They were unsuccessful, however, due to the difficulty of handling such an apparatus on a laboratory scale. This method was used successfully, however, in a large fermenter of 4,000 liters capacity. These experiments will be described elsewhere.[10]

[10] Northrop, J. H., Ashe, L. H., and Morgan, R. R., *J. Ind. and Eng. Chem.*, 1919 (in press).

SUMMARY.

1. An organism has been described which produces acetone and ethyl alcohol with smaller amounts of higher alcohols from starch or sugar.

2. The optimum conditions of temperature, reaction of the media, physiological state of the culture, size of inoculation, air supply, and nitrogen supply have been determined.

3. A semicontinuous method for carrying on the fermentation has been described.

The authors wish to acknowledge their indebtedness to Dr. P. A. Levene for many helpful suggestions during the course of the work.

THE DETERMINATION OF β-HYDROXYBUTYRIC ACID, ACETOACETIC ACID, AND ACETONE IN BLOOD.

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(Received for publication, June 21, 1919.)

In the determination of acetone bodies in the blood as described by us,[1] we neglected to state that the precipitate should be filtered soon after the period of boiling is ended. If the mixture is allowed to cool and stand for some hours, several mg. of flocculent precipitate of indefinite origin may form and cause a plus error of appreciable magnitude in the results.

[1] Van Slyke, D.D., and Fitz, R., Studies of acidosis. VIII. The determination of β-hydroxybutyric acid, acetoacetic acid, and acetone in blood, *J. Biol. Chem.*, 1917, xxxii, 495.

STUDIES OF THE GASTRIC RESIDUUM.

III. AMINO-ACID NITROGEN.

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(Received for publication, June 21, 1919.)

The determination of amino-acid nitrogen in the stomach contents has received but very little attention. Carlson (1) reports figures of amino-acid nitrogen obtained from the hunger juice of two subjects by the formol titration method. He found it to vary from 3 to 9 mg. per 100 cc. No results were reported on the so called "appetite juice."

Zunz (2) determined the amino-acid nitrogen in test meals obtained from normal and pathological cases. These meals were removed at the one hour period. He reports 10 mg. per 100 cc. of gastric content as the maximum in normal cases and calls attention to the fact that in gastric cancer considerably higher values were obtained.

The results of these investigators, however, are not directly comparable to those reported in this paper, as the methods of collection and the material worked upon are quite different.

Methods.

The subjects of the experiments were twenty-five apparently normal young women students in physiological chemistry at Iowa State College, and one apparently normal man (Subject 120).

The residuums were obtained by means of the Rehfuss gastroduodenal tube according to methods previously described (3). The volumes and specific gravities were determined and the physical characteristics were noted immediately. The determinations of total acidity, free acidity, peptic and tryptic activity,

and amino-acid nitrogen were made as soon as possible upon the samples.

The methods for the determination of total acid, free acid, pepsin, and trypsin were performed according to the directions of Rehfuss, Bergeim, and Hawk (4), with the exception of pepsin which was incubated 14 hours instead of 12. Amino-acid nitrogen was determined by the procedure of Van Slyke (5).

TABLE I.

Subject No.	Description of sample.	Volume.	Total 0.1 N acid per 100 cc.	Free 0.1 N acid per 100 cc.	Pepsin.	Trypsin.	Specific gravity.	Amino-acid nitrogen per 100 cc.
		cc.	*cc.*	*cc.*	*mm.*			*mg.*
120	Colorless	23	38.0	28.0	6.3	2	1.010	17.14
121	Some saliva, colorless	17	20.0	12.0	4.0	0	1.010	23.22
122	Viscous	33	28.0	21.0	6.3	0	1.0071	12.83
123	Colorless	27	35.0	26.0	4.0	16	1.0067	21.84
124	Saliva, yellow	74	35.0	22.0	9.0	0	1.0067	29.07
125	Colorless	46	45.0	41.5	6.3	16	1.0059	22.49
126	Green	45	13.0	0	2.0	0	1.0075	32.64
127	Yellow mucus	25	10.0	0	0	0	1.0071	39.71
128	Colorless	45	65.5	51.0	2.8	0	1.0091	22.06
130	Light green	35	78.0	67.5	9.0	2	1.0074	43.20
131	Colorless saliva	35	73.0	61.0	6.5	16	1.0039	27.58
132	Light green	35	35.0	14.0	2.8	16	1.0040	33.09
133	Yellow, some blood	33	36.0	22.0	3.1	16	1.0049	37.80
134	Colorless	83	50.0	29.0	4.0	2	1.0057	67.26
135	"	30	9.0	0	0	1	1.0074	39.24
137	Light yellow	35	41.0	13.0	2.3	2	1.0081	20.52
138	Colorless	22	39.3	10.0	5.3	1	1.0050	46.64
139	"	65	68.0	45.0	4.0	1	1.0061	21.88
140	Some saliva	40	88.0	47.0	4.5	2	1.0055	21.83
141	Colorless	27	39.4	15.0	4.3	1	1.0088	22.76
142	Light green	5	88.0	7.2				38.19
143	Colorless	15	31.5	10.0	2.5	0	1.0082	38.18
144	"	23	42.25	15.5	4.0	0	1.0045	27.27
146	Mucus colorless	35	65.0	46.5	5.3	2	1.005	29.83
149	Some bile	28	47.0	33.5	0	2	1.0040	48.82
150	" "	50	50.0	22.5	0	8	1.0054	43.40

DISCUSSION.

The values of total acidity, free acidity, pepsin, and trypsin were found to give about the same average and variations therefrom as reported in a previous investigation (3). The average volume, however, was found to be much lower, 35 cc. against 49 cc. reported in that paper. The smallest quantity was 5 cc. and the largest 83 cc. The specific gravity averaged 1.006 which compares favorably with the average determined by Fowler, Rehfuss, and Hawk, which was 1.0056 (100 cases) (6).

The amino-acid nitrogen averaged 32.6 mg. per 100 cc. The lowest figure was 17.14 mg. and the highest 67.26 mg. Attention should be called to the fact that these values are considerably higher than the values reported by Carlson, even our lowest value being much greater than his maximum. The high values which were obtained might seem to invalidate the significance of the high values, reported by Zunz, in cancer cases (2).

Had our gastric residuum been diluted to the approximate quantity of a test meal the results would have been somewhat the same. The high amino-acid content, in some cases, is probably due to regurgitated material from the intestine.

The amino-acid nitrogen apparently bears no relationship to the free or total acidity or to the pepsin or trypsin content. This finding is in accordance with Zunz's results on test meals.

SUMMARY.

1. The average amino-acid nitrogen found in twenty-six normal cases of gastric residuum was 36.4 mg. per 100 cc.

2. The amount of amino-acid nitrogen apparently bears no relationship to acidity or pepsin or trypsin content.

3. In view of our data the high amino nitrogen value of the contents of the fasting stomach appears not to be significant in gastric carcinoma.

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NUTRITIVE FACTORS IN PLANT TISSUES.

II. THE DISTRIBUTION OF WATER-SOLUBLE VITAMINE.*

PRELIMINARY REPORT.

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(Received for publication, June 9, 1919.)

Experimental evidence is rapidly accumulating to show the wide-spread distribution of the indispensable food factor known as water-soluble vitamine in naturally occurring food products. Its presence in the seeds of cereals and of a number of legumes is now familiar; and recent studies have indicated that certain other vegetable products also may serve as sources of this vitamine. Among them are cottonseed,[1,2,3] millet seed,[1] flaxseed,[1,4] Kafir corn,[4] hempseed,[1] cabbage,[1,5] alfalfa,[1,5,6] clover,[1,5] timothy,[5] spinach,[5] potato,[7] carrots,[8] and coconut meal.[9]

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

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[9] Johns, C. O., Finks, A. J., and Paul, M. S., *J. Biol. Chem.*, 1919, xxxvii, 497.

To this list we can now add the bulb of the *onion*, the root of the *turnip*, the leaves, stem, and roots of the *beet*, and the fruit of the *tomato*—foods representing unlike parts as well as genera of plants. Chart I shows a few illustrative instances of the restoration of growth in white rats through addition of these vegetables products in dried form to a ration, devoid of water-soluble vitamine, upon which the animals would inevitably fail to grow and would decline in weight.

In order that we may make intelligent and economical use of the various foods to insure an adequate supply of the water-soluble vitamine, it is important to know not only how widely this essential food factor is distributed, but also how the different natural foods compare with each other as sources of this accessory. It has generally been supposed that the fruits and vegetables are useful in the dietary solely because of the inorganic salts, organic acids, and roughage which they supply. Now that some of these plant products have been found to be relatively rich in their content of vitamines we may revise our estimate of their value in nutrition.

The method ordinarily used in studying the vitamine content of various food substances has been to incorporate a certain percentage of the product to be tested in the basal, vitamine-free ration. As has been pointed out in a previous communication[5] this method gives no definite indication of the actual quantity of the vitamine-containing substance needed for adequate nutrition, unless the food intake is known so that the real consumption of the product tested can be calculated therefrom. Although by this method it is possible to obtain an approximate idea of the amount necessary, it is difficult to learn definitely what is the actual minimum required. Inasmuch as a young rat during the earlier stages of growth normally eats increasingly larger amounts of food, if this contains a fixed percentage of the substance to be tested the animal will gradually increase its consumption of vitamine in proportion as its food intake increases.

We have therefore endeavored to apply a method which will permit more accurate comparisons of the water-soluble vitamine content of natural foods. The procedure consisted in feeding each day small, known quantities of the vegetable product under investigation apart from the basal ration which was fed *ad libitum*.

In this way the effects of feeding similar quantities of the different food substances could be compared, and a fairly accurate estimate of their relative value in nutrition, based on their content of water-soluble vitamine, could be obtained.

The results with the water-soluble and also the fat-soluble vitamine will be presented in detail in a later publication. In addition to the purely qualitative features already discussed we desire to point out here a striking feature discovered in relation to the maturity of the plants used in some of our experimental series. Hitherto no attention has been given to the effect of this factor upon the content of vitamine in plants. To test this point we secured samples of clover, alfalfa, and timothy hay from the same fields from which the *immature* products were obtained earlier in the season. When the hays were used to furnish the water-soluble vitamine they were found to be much less efficient than the dried immature specimens (Chart II). This difference may be due to the larger proportion of inactive structural tissue in the mature plants. The fact that such a difference exists may have importance in feeding young animals and dairy cows. If the beneficial result of milk in calf feeding is due, in part at least, to its content in water-soluble vitamine, it may be that hay made from immature clover or alfalfa can be used to replace a part at least of the milk fed to young stock. Furthermore, if the vitamine content of milk bears any relation to the vitamine content of the food of the cow the water-soluble vitamine of the milk may be increased perhaps by replacing the hay ordinarily used with that made from less mature plants. Heretofore it has been considered to be important to cut hay when the plant has reached the stage where the proportion of digestible nutrients is at the maximum.[10] Since the small quantity of digestible nutrients supplied by hay can be furnished readily by concentrates the question arises whether, or not, a greater nutritive advantage lies in considering primarily the nutrient content or the comparative richness in vitamine.

[10] For a discussion of current views on this subject see Henry, W. A. and Morrison, F. B., Feeds and feeding, Madison, 15th edition, 1915.

CHART I. Restoration of growth after addition of dried vegetable products to a ration, devoid of water-soluble vitamine, upon which the animals had previously declined in weight.

The composition of the basal ration was as follows:

Rats 5104, 5118, 5120.	*per cent*	Rats 5039, 5205, 5206.	*per cent*
Meat residue	19.6	Casein	18.0
Salt mixture*	4.0	Salt mixture*	4.5
Starch	52.4	Starch	50.5
Butter fat	9.0	Butter fat	9.0
Lard	15.0	Lard	18.0

* The composition of the salt mixture used is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

The plant product was fed either separately and apart from the basal ration, or incorporated in the food, in which case it replaced an equivalent amount of starch.

The figures on the curves represent the quantities of plant tissue fed in gm. per day (apart from the basal ration) or percentages incorporated in the food and their equivalent in gm. per day.

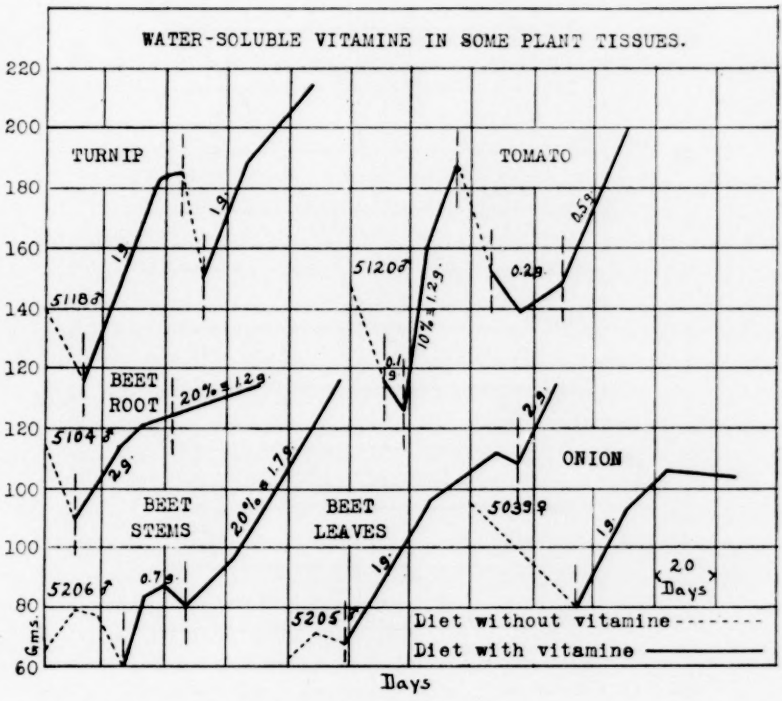
WATER-SOLUBLE VITAMINE IN SOME PLANT TISSUES.
220
200
180
160
140
120
120
100
100
80
60
Gms.
TURNIP
TOMATO
BEET ROOT
BEET STEMS
BEET LEAVES
ONION
5118 g
5104 g
5206 g
5205 g
5120 g
5039 g
1 g
2 g
0.7 g
0.1 g
0.2 g
0.5 g
20% @ 1.2 g
20% @ 1.7 g
10% @ 1.2 g
20 Days
Diet without vitamine
Diet with vitamine
Days

Chart I.

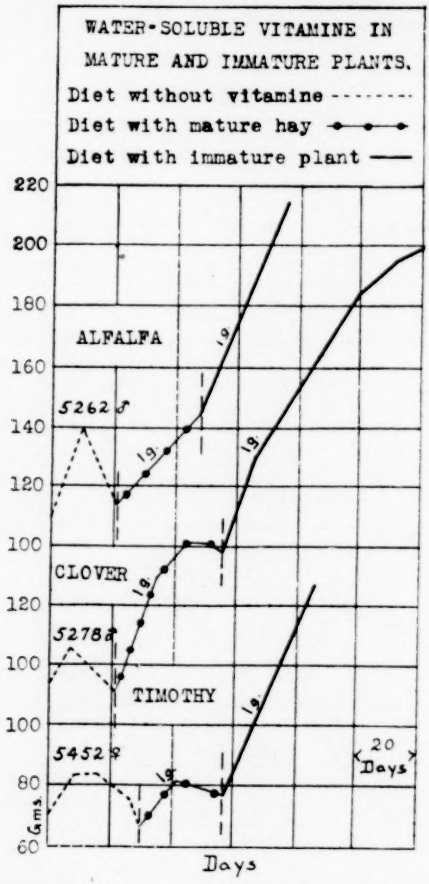

CHART II. Contrasting the efficiency of dried *mature* and *immature* preparations of the same plant as sources of water-soluble vitamine with a ration devoid of this food accessory.

The composition of the basal ration was as follows:

	per cent
Meat residue	19.6
Salt mixture*	4.0
Starch	52.4
Butter fat	9.0
Lard	15.0

* The composition of the salt mixture used is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

When the plant product was used as a source of vitamine 1 gm. per day was fed apart from the basal ration.

PREPARATION OF PROTEIN FREE FROM WATER-SOLUBLE VITAMINE.*

By THOMAS B. OSBORNE, ALFRED J. WAKEMAN, AND EDNA L. FERRY.

(*From the Laboratory of the Connecticut Agricultural Experiment Station, New Haven.*)

(Received for publication, June 9, 1919.)

There is at present no criterion whereby the complete absence of vitamine from any of the ingredients of the diet can be proved, because no one knows how long an animal can live nor how rapidly it will decline in body weight on a diet absolutely free from vitamine. A great many experiments have established the fact that animals do not live long and that they soon begin to decline in body weight when fed on diets from which the water-soluble vitamine has been removed by the current methods.

All investigators now appear to be convinced that the diets of purified foodstuffs used for some of the earlier experiments in which good growth was reported were not so free from water-soluble vitamine as they were then thought to be. Inasmuch as the chemical nature of vitamines is wholly unknown the efficiency of chemical methods of purification can be learned only from the results of feeding experiments. Accordingly we have attempted to determine the relative efficiency of the various methods used for removing the water-soluble vitamine from preparations of proteins of different kinds. This has been done by comparing the length of life, or rate of decline, of young rats fed on diets containing one protein or another, together with a basal ration supposedly free from water-soluble vitamine. All the ingredients of the latter were taken from the same stock supply so that the components of the several diets were alike except for the protein.

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

Previous experiments have shown that after the addition of water-soluble vitamine all these diets were adequate for normal growth, except the one containing gliadin, and this diet sufficed for long continued maintenance. The proteins were so chosen as to represent a wide variety in chemical constitution as well as in the conditions employed in extracting them from the tissues and in subsequently purifying each preparation. The methods used were the following.

Crude Edestin.—Clean hempseed was extracted by grinding with 10 per cent sodium chloride solution and the extract mixed with paper pulp, pressed out with a hydraulic press, and filtered clear through a bed of paper pulp. The extract was then diluted with four volumes of warm water, the final temperature being about 40°. On cooling the edestin separated in crystals from which, after settling, the solution was syphoned and the crystals collected on a filter. These were redissolved in salt solution and again crystallized as before. The recrystallized edestin was then digested in alcohol of about 50 per cent by volume, washed with the same until the washings were free from chlorides, digested with strong alcohol, collected on a filter, and dried in the air.

Pure Edestin.—This was prepared by recrystallizing the crude preparation five times more.

Cottonseed Globulin.—Cottonseed meal was extracted with 10 per cent sodium chloride solution and the extract filtered through paper pulp and poured into about four volumes of water. After settling the deposit was dissolved in salt solution, filtered through paper pulp, and reprecipitated by pouring into several volumes of water. After settling the precipitate was washed with 50 per cent alcohol till the washings ceased to react for chlorides, and then digested twice with 95 per cent alcohol and dried in the air.

Ovovitellin.—Egg yolks were mixed with an equal volume of 10 per cent sodium chloride solution and shaken with an equal volume of ether in a separatory funnel. The aqueous layer was again shaken with ether and then filtered through paper pulp. The clear solution was dialyzed for 6 days. The precipitate thus produced was collected on filters and, after draining over night, was dissolved in salt solution, filtered through paper pulp, and reprecipitated by dialysis. After collecting on filters the pre-

cipitated vitellin was digested twice with 95 per cent alcohol, twice with absolute alcohol, once with ether, and was then air-dried.

Lactalbumin.—The filtrate from casein which had been precipitated by adding dilute hydrochloric acid to skimmed milk was heated to boiling and the coagulum filtered out, washed chloride-free with boiling water, digested with dilute alcohol, washed with 50 per cent alcohol until lactose was removed, then digested with strong alcohol, pressed in a hydraulic press, ground to a powder, and dried in the air.

Gliadin.—Thoroughly washed wheat gluten was extracted with hot 70 per cent alcohol, the extract filtered clear, concentrated to a syrup, and the gliadin precipitated by pouring into a large volume of ice water. The precipitate was dissolved in warm 70 per cent alcohol and the clear solution poured into a large excess of strong alcohol. The precipitated gliadin was then digested with absolute alcohol and afterwards with ether, ground to a powder, and dried in the air.

Pure Casein.—Centrifugated milk was treated with very dilute hydrochloric acid until a sharp separation resulted. The precipitate was collected on cheese-cloth, the milk serum removed as completely as possible with hydraulic press, and the cake ground up with water and dissolved by adding dilute sodium hydroxide solution, care being taken to avoid an excess of alkali. The casein was then reprecipitated three times with dilute acetic acid and finally, after filtering its alkaline solution through paper pulp, again precipitated with dilute hydrochloric acid. The casein was then digested with 50 per cent alcohol and washed therewith until the washings were free from chlorides, then with strong alcohol, and dried in the air.

Very Crude Casein.—Separator milk was precipitated with dilute hydrochloric acid, the casein strained out on cheese-cloth, pressed in hydraulic press, ground in Nixtamal mill with water, again pressed, and then dried in a current of warm air.

Meat Residue.—Fresh lean round of beef was run through the meat chopper, ground to a paste in a Nixtamal mill, stirred into twice its weight of water, and boiled for a few minutes. The solid residue was then strained on cheese-cloth, pressed in the hydraulic press, and the cake stirred into a large quantity

of boiling water. After repeating this process of washing with hot water, the extracted residue was dried rapidly in a current of warm air at about 60°.

The considerable differences shown in Table I in the percentages of lard in these foods were made necessary by the physical condition of the several preparations of protein. When these were light and powdery larger proportions of fat were required to make a mixture sufficiently coherent to avoid losses due to scattering by the animals.

TABLE I.

Composition of the Food Mixtures.

	Edestin.		Lactalbumin.	Cottonseed globulin.
	Pure.	Crude.		
	per cent	*per cent*	*per cen*	*per cent*
Protein	18.0–36.0	18.0	18.0	18.0–36.0
Starch	46.5–28.5	46.5	41.0	47.0–29.0
Lard	22.0	20.0	27.0	21.0
Butter fat	9.0	9.0	9.0	9.0
Salts*	4.5	4.5	5.0	5.0
	Gliadin.	Ovovitellin.	Casein.	Meat residue.
	per cent	*per cent*	*per cent*	*per cent*
Protein	18.0	18.0	18.0	19.6
Starch	50.0	50.0	50.0	52.4
Lard	18.5	18.5	18.0	15.0
Butter fat	9.0	9.0	9.0	9.0
Salts*	4.5	4.5	5.0	4.0

* The composition of the salt mixture employed is given in Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

Table II shows the gains and losses made by the rats fed on these diets and the chart reproduces these data in graphic form.

These figures show that on the diets containing lactalbumin, gliadin, ovovitellin, casein, pure or crude, or meat residue, nearly all the rats lost from 30 to 40 per cent, or even more, in weight, and all but one of them died within 50 days unless water-soluble vitamine was supplied earlier. When yeast was given to some of the rats after they had declined severely all recovered promptly, thus demonstrating that their failure to be maintained was due to a lack of this factor in their previous diet.

TABLE II.

Rat No.	Source of protein.	Original body weight.	Loss or gain.	Time.	Result.
		gm.	*gm.*	*days*	
5160	Edestin, crude.	100	−50	72	Died
5161	" "	102	− 7	104	Diet changed.
5167	" "	100	+10	104	" "
5310	" pure.	106	−37	30	" "
5312	" "	110	−35	30	" "
5306	" "	110	−36	30	" " Died a few days later.
5505	" "	1[illegible]5	−51	52	Died.
5506	" "	120	−52	49	"
5497	" "	114	−58	40	"
5162	Cottonseed globulin.	107	± 0	104	Diet changed.
5163	" "	120	− 5	104	" "
5164	" "	115	−15	104	" "
5159	Lactalbumin.	90	−48	45	Died.
5165	"	1[illegible]2	−63	58	"
5166	"	113	−49	41	"
5169	"	100	−50	40	"
5170	Gliadin.	9[illegible]	−40	42	"
5171	"	100	−50	48	"
5174	"	106	−37	41	"
5173	Ovovitellin.	106	−37	42	Diet changed.
5175	"	90	−42	39	Died (infected lungs).
5172	"	96	−26	42	Diet changed.
5039	Casein, pure.	115	−35	31	" "
5079	" "	113	−23	31	" "
5058	" "	90	−30	32	Died.
5477	" "	115	−46	75	Died (chloroformed because nearly dead).
5489	" "	116	−65	64	Died.
5492	" "	115	−57	45	"
5555	" very crude.	66	−13	42	"
5530	" " "	65	−22	38	"
5575	" " "	65	−28	42	"
5054	Meat residue.	110	−37	31	Diet changed.
5064	" "	110	−30	31	" " Died a little later.
5070	" "	125	−43	34	Died.
5475	" "	115	−43	62	"
5488	" "	112	−50	71	"
5493	" "	120	−56	54	"
5458	" "	66	−27	48	"
5459	" "	54	−18	47	"
5460	" "	75	−38	43	"

In contrast with these rats which, with only one exception, failed so rapidly, those having crude edestin, or cottonseed globulin, were maintained during 3 months without notable change in body weight. When the amount of protein was doubled all but one of these rats gained weight fairly rapidly, presumably because the amount of vitamine supplied by the crude protein was also doubled. When edestin, purified by seven recrystallizations, was fed, three of the rats lost over 30 per cent of weight within a month, and recovered on supplying water-soluble vitamine, while the other three died in from 40 to 46 days, after losing about 40 per cent in weight. Although the crude edestin had been twice crystallized from a large volume of dilute salt solution, and the cottonseed globulin had been twice precipitated by diluting its saline solution with a very large volume of water, and both were then thoroughly washed with 50 per cent alcohol (in which the water-soluble vitamine is much more soluble than in stronger alcohol), and next extracted with 95 per cent alcohol, both still contained water-soluble vitamine.

The persistence with which this vitamine is retained by edestin suggests that it is chemically combined therewith. In this respect it resembles acids which combine with edestin to form salts. Thus, if edestin is crystallized from sodium chloride solutions it separates as a hydrochloride. When edestin hydrochloride is recrystallized from a solution of a sulfate it separates as a mixture of sulfate and hydrochloride. On further recrystallization the proportion of chloride diminishes, so that after a sufficient number of crystallizations no more hydrochloride remains.

The repeated crystallizations required to remove the water-soluble vitamine from edestin are analogous to those required to remove chlorine by recrystallization from sulfate solutions. Perhaps in this phenomenon we have a hint that the water-soluble vitamine may have acid properties, notwithstanding the fact that it has heretofore been regarded as of probably basic character, presumably because it is precipitated by phosphotungstic acid. Although yeast nucleic acid is a relatively strong acid it is precipitated by this reagent. (Communicated by Dr. C. O. Johns.)

The results obtained with casein are of interest because the

CORRECTION.

On pages 41 and 42, Vol. XXXIX, No. 1, August, 1919, line 1, page 42, should precede line 1 of page 41.

but the results of their experiments show ... of the vitamine was retained in the preparation of this protein coagulated in an acid solution.

The insignificant quantity of the water-soluble vitamine present in patent flour[1,2] explains its absence from the preparation of gliadin.

The relatively large amount of phosphorus in the ovovitellin, greater even than in casein, suggests that this protein may have acid properties similar to those of casein and consequently may be unable to enter into chemical combination with the water-soluble vitamine. However this may be, the results of our experiments show that under the conditions of preparation of the ovovitellin this food factor was removed.

The freedom of the meat residue from the water-soluble vitamine may be ascribed to the small amount present in muscle tissue.[3] Meat residue, prepared by the method described in this paper, furnished a comparatively cheap source of protein for experiments requiring a basal diet free from water-soluble vitamine for, even if carelessly prepared, there is little danger that this product will contribute an appreciable amount of this factor to the diet.

Whether any of these diets are actually free from water-soluble vitamine cannot be asserted, but the fact that, irrespective of the source of protein, all the rats except those on the cottonseed globulin or crude edestin died within approximately the same length of time and with about the same loss of weight

[1] Voegtlin, C., Lake, G. C., and Myers, C. N., *Public Health Rep.*, 1918, xxxiii, 647.

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

[3] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 309.

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The results obtained with casein are of interest because the

on the carefully purified casein. Contrary to all that has been believed in the past it appears that little, if any, water-soluble vitamine is present in unpurified casein. In view of the ease with which, under some conditions, the antineuritic vitamine is adsorbed it is surprising that so little of the water-soluble vitamine is adsorbed by casein thus precipitated from undiluted milk. That apparently little, if any, is present in the crude casein possibly may be due to the acid character of this protein. Very little is known of the basic or acid properties of lactalbumin but the results of these experiments show that practically none of the vitamine was retained in the preparation of this protein coagulated in an acid solution.

The insignificant quantity of the water-soluble vitamine present in patent flour[1,2] explains its absence from the preparation of gliadin.

The relatively large amount of phosphorus in the ovovitellin, greater even than in casein, suggests that this protein may have acid properties similar to those of casein and consequently may be unable to enter into chemical combination with the water-soluble vitamine. However this may be, the results of our experiments show that under the conditions of preparation of the ovovitellin this food factor was removed.

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[1] Voegtlin, C., Lake, G. C., and Myers, C. N., *Public Health Rep.*, 1918, xxxiii, 647.

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

[3] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 309.

rats fed on the crude preparation died almost as soon as those makes it probable that the protein components of these diets did not contribute appreciable quantities.

If any of the other constituents of the diet contained traces of the water-soluble vitamine only the starch requires consideration, because it seems fair to assume that neither the fats nor the inorganic salts contain any. In respect to starch there is as yet no conclusive evidence beyond the fact that this can at most contain only traces.

The slight initial gain in weight by many of the animals, as shown in the chart, must be attributed to a store of vitamine in the animal itself, for when this is depleted by previously feeding with diets low in vitamine a fall in weight immediately follows a return of the animal to the vitamine-free diet. This is well illustrated by Chart II in an earlier paper[3] and has been confirmed by a very large number of later observations.

These experiments show that vigorous young rats of about 100 gm. initial weight, when fed on diets as free from water-soluble vitamine as were these, usually die within 50 days with an average loss of body weight of about 45 per cent. This is a distinctly greater loss of weight than the average of 37 per cent observed when no food was supplied. On the other hand the duration of life was much longer than when no food was given, which is about 4 days for small and 7 days for larger rats.[4]

Recently Voegtlin and Lake[5] have used ground beef muscle treated with 10 per cent sodium carbonate solution until the tissue reacted strongly alkaline to litmus and then heated at 120° under pressure for 3 hours. In view of the frequent assertion that alkali destroys the water-soluble vitamine these authors assumed that their preparation of meat was free from this food factor. Six rats whose sole food consisted of meat thus treated lived for more than 3 months and all gained in weight, one even living for about 7 months and gaining about 150 per cent in weight. In view of the results which we obtained[3] with meat powder it is difficult to imagine the source of the water-soluble vitamine in Voegtlin and Lake's diet. Dried lean round of beef

[4] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxix, 289.

[5] Voegtlin, C., and Lake, G. C., *Am. J. Physiol.*, 1918–19, xlvii, 558.

furnishes so little of this vitamine that animals fail almost as quickly when this is the sole source of protein as when the extracted meat residue is used.[3] If Voegtlin and Lake's results are contrasted with those which we have obtained by the use of meat which was extracted thoroughly merely with boiling water it is difficult to believe that the treatment with alkali which Voegtlin and Lake employed did in fact destroy all the water-soluble vitamine in the meat they used. That the water-soluble vitamine is more resistant to alkali than has been commonly supposed is indicated by the following experiment.

Finely ground dry brewery yeast was digested with ten times its weight of 0.1 N sodium hydroxide solution for 21½ hours and then heated on a boiling water bath for 2 hours. The alkali was then neutralized to litmus with hydrochloric acid and the yeast dried in a current of warm air at about 60°. When 0.2 gm. of this yeast was fed to rats which had declined on a diet practically free from water-soluble vitamine recovery was as prompt as when the untreated yeast was used. If the water-soluble vitamine had been destroyed by the alkali the destruction did not proceed to such an extent as to be detected by these experiments. Less than 0.2 gm. of the untreated dried yeast is usually too little to induce such prompt recoveries as were observed in these cases.

Not only the results of this present investigation, but also many of those reported by others who have used supposedly vitamine-free proteins for their experimental diets, show how difficult it may be to obtain preparations actually free from this dietary factor. It does appear, however, that many proteins, especially casein, can easily be prepared so free from the water-soluble vitamine that animals decline in weight within a few days, unless some other source of this food factor is supplied.

It is difficult to understand why so many instances are recorded in the literature of rats which have maintained their body weights for many weeks when fed on diets which, in view of our present state of knowledge, should have been very nearly free from the water-soluble vitamine. Thus to refer to recent publications we are at a loss to explain why Voegtlin and Lake's rats throve so well on their meat diet. Furthermore, in a recent paper by McCollum and Simmonds[6] are many charts in which

[6] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 55.

during Period 1 a food containing casein as its sole protein was fed. This diet the authors state "was free from water-soluble B." It is to be noted, however, that none of their rats lost weight during the 4 to 6 weeks of this period, and many of them gained not a little. This is in marked contrast to the behavior of our rats on *crude* casein, all but one of which began to lose weight within 10 days and were near death with large loss of weight in 30 to 40 days. In view of the rapid decline of our rats on the *crude* casein it seems improbable that the casein used by McCollum and Simmonds supplied any appreciable amount of the water-soluble vitamine.

Our present experiments appear to demonstrate conclusively that animals begin to decline within a few days on a diet nearly, if not quite, free from this vitamine. Therefore we conclude that in those experiments which are recorded in the literature where animals failed to lose weight for some weeks water-soluble vitamine was supplied from some unrecognized source.

As a result of Stepp's early experiments extraction with strong alcohol has been relied on to remove the water-soluble vitamine from preparations of protein and this method still is employed. It is to be noted that rats failed rapidly on the meat residue and also on the crude casein diets, none of the ingredients of which ever had been treated with alcohol. Extraction with alcohol therefore is not essential for the production of protein free from the water-soluble vitamine.

We have long known that the water-soluble vitamine is insoluble in absolute alcohol[7] and that its solubility in "alcohol" depends on the amount of water this contains. It is doubtless due to this fact that investigators have obtained such discordant results when using proteins thus "purified," for it seems almost certain that preparations thus treated would retain more or less of the water-soluble vitamine according to the amount of water present when the alcohol was applied and also to the manner of its application. For a long time past, after washing with water, we have depended on a thorough extraction with 50 per cent alcohol to remove this vitamine before the treatment with strong alcohol. Because many proteins are converted into a colloidal

[7] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 149.

state by washing with distilled water before chlorides are completely removed, we have used 50 per cent alcohol to complete the removal of soluble substances.

That even extensive extractions thus conducted, together with great care to secure very fine subdivision of the protein during extraction, may fail to remove all the vitamine is plainly shown by the results obtained with the "crude" edestin and cottonseed globulin. These preparations ordinarily would be considered pure, for under the conditions of their production it is difficult to believe that they contained more than minimal traces of water- or alcohol-soluble impurities, unless these are chemically combined. It is possible that the vitamine may combine more firmly with these two proteins which are relatively rich in basic amino-acids than with the others used in the experiments here described.

It is evident that unless the diet is proved to be free from the water-soluble vitamine by first demonstrating its failure to maintain the body weight and health of young animals, it cannot be considered suitable for use in feeding experiments designed to show the proportion of water-soluble vitamine in any other element of the food. Since we have no other criterion than the above by which the success of the purification methods can be judged, we cannot consider the diet to be vitamine-free unless the animals fail at least as rapidly as did those recorded in the chart which were fed on meat residue or casein. It is, of course, impossible to assert that the materials in these experiments did not still contain enough vitamine to prolong the lives of the animals somewhat. Although these diets may not have been absolutely vitamine-free they are pure enough for use in studying many of the problems connected with the influence of the water-soluble vitamine on nutrition, or the presence or relative proportion of this factor in our various food products.

In regard to the carbohydrates to be used in making diets free from water-soluble vitamine the confusion introduced into the earlier literature by the now demonstrated presence of this factor in commercially "pure" lactose need no longer concern us, because the difficulties thus occasioned can be avoided by using dextrin or starch. From the experiments described in this paper it is evident that the commercial corn-starch, which we

used, contained no appreciable quantity of the water-soluble vitamine; for even though this starch constituted about 50 per cent of the diets the rats died after a short period. That starch furnishes carbohydrate in a form adequate for the normal nutrition of rats has been demonstrated by hundreds of experiments which have been made in this laboratory, which fact, in connection with the ease with which the commercial starch, practically free from water-soluble vitamine, can be obtained, makes this an excellent source of carbohydrate for vitamine-free diets.

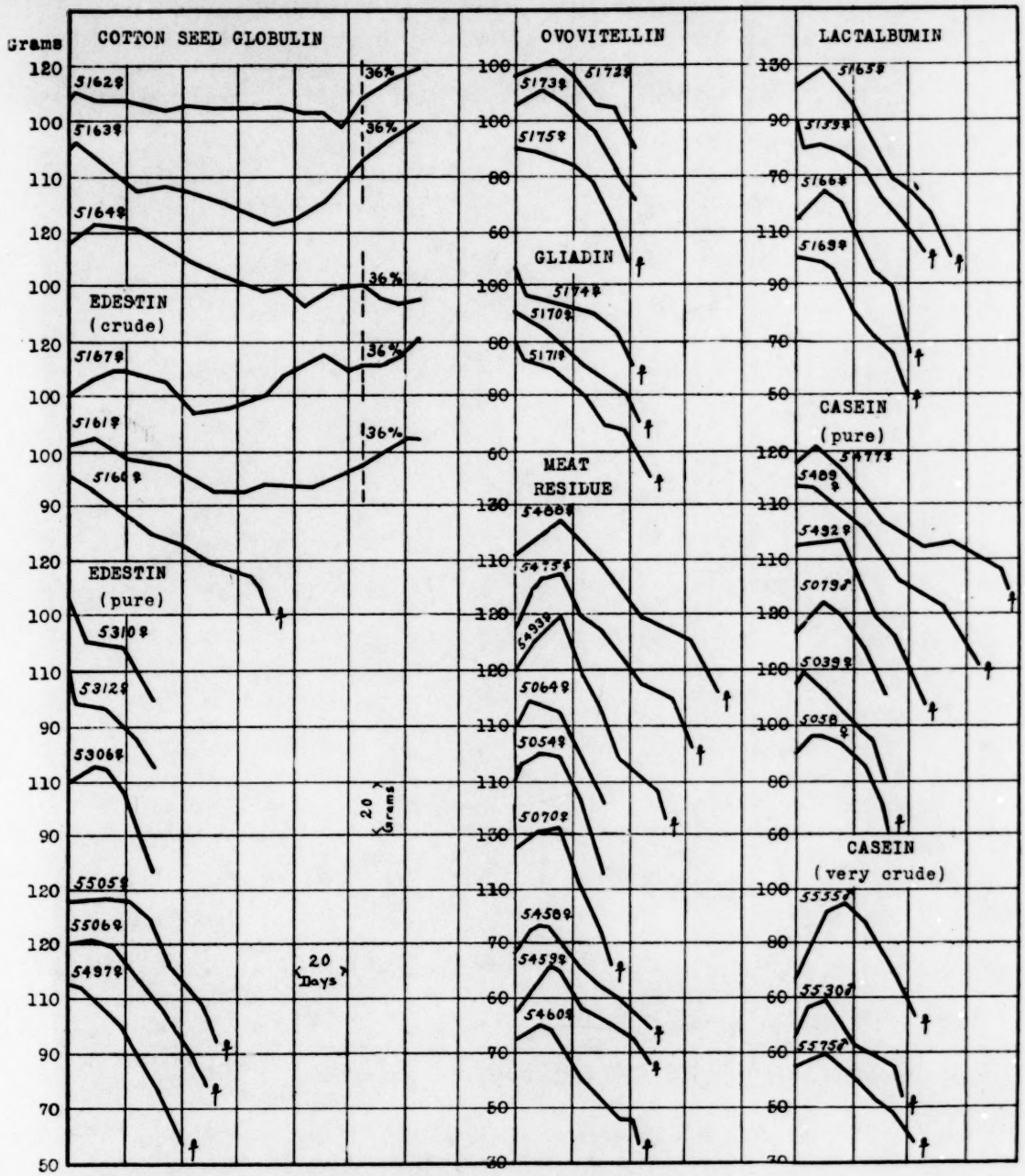

CHART I. Body weight graphs of young albino rats fed on diets supposedly free from water-soluble vitamine except in so far as this is supplied by the protein indicated on the chart. The food mixtures used are given on page 38; the methods used for preparing the proteins on pages 36 and 37.

NON-PROTEIN NITROGENOUS CONSTITUENTS OF HUMAN MILK.

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Although the presence in milk of "extractives" has been recognized for many years, studies on this phase of the milk question have been relatively few, and have, in most cases, dealt with the occurrence of a single member of the group such as ammonia, urea, uric acid, etc.[1]

In this paper we wish to report the results of a series of observations made on the milk of normal women in which we have made determinations of total non-protein nitrogen, urea, uric acid, creatine, and creatinine, in an effort to obtain data regarding the relative quantities of these substances present under normal conditions. In a later paper it is our intention to present similar data on pathological material.

The analytical methods used were described in a paper recently published from this laboratory.[2] All milk samples analyzed were obtained from normal women whose infants were free from digestive disturbances, and were examined within a few hours after being drawn.

Before attempting the collection of a large number of samples, it seemed desirable to determine whether the output of extractives was more or less constant throughout the 24 hours, in other words, whether the time of day at which the sample was collected must be considered in the interpretation of the analyses.

[1] Raudnitz, R. W., *Ergebn. Physiol.*, 1903, ii, pt. 1, 255, contains a bibliography of the literature up to 1902. Later references on the subject are as follows: Filia, A., *Z. Biochem. Biophys.*, 1912, xii, 425. Sherman, H. C., Berg, W. N., Cohen, L. J., and Whitman, W. G., *J. Biol. Chem.*, 1907, iii, 171. Voegtlin, C., and Sherwin, C. P., *J. Biol. Chem.*, 1918, xxxiii, 145.

[2] Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1919, xxxvii, 353.

In Table I are presented the results of the examination of thirty-one samples of milk from six wet-nurses. These samples were obtained at 3 hour intervals during the day, from 6 a.m. to 9 p.m. In each instance the breast was pumped until no more milk could be obtained, the product was mixed, measured, and a 60 cc. specimen sent to the laboratory. From these results it is apparent that while there are slight variations in the amount of the various extractive substances present in samples of milk drawn at different periods throughout the day, these variations are relatively small, and could not be considered of importance in the interpretation of results. There is also no apparent uniformity in the slight variations noted, in some cases for instance the highest value for urea was found in the samples drawn during the forenoon, in others a maximum was attained in the late afternoon or evening.

Table II contains results obtained by the examination of 40 single specimens of milk taken from as many women.

The results of the examination of these 71 samples of milk may be summarized as follows:

	Minimum. *mg.*	Maximum. *mg.*
Total non-protein nitrogen	20.0	37.0
Urea nitrogen	8.3	16.0
Amino nitrogen	3.0	8.9
Preformed creatinine	1.0	1.6
Creatine	1.9	3.9
Uric acid	1.7	4.4

Those familiar with modern blood analysis will immediately recognize the fact that the values found for non-protein nitrogen, urea, preformed creatinine, and uric acid in milk are along the same general level as the figures obtained for the same substances in blood. The concentration of amino nitrogen is in some cases comparable with the amount of this substance in blood, but in other samples sinks to a considerably lower lever, while the values for creatine are also much lower than in blood. In view of these findings, we have in twenty cases made determination of non-protein nitrogen and urea in blood and milk. The results of this work are presented in Table III. An inspection of these figures indicates so clearly the close relation between the concentration of non-protein nitrogen and urea in blood and milk that further comment would seem unnecessary.

TABLE I.

Non-Protein Nitrogenous Constituents of Human Milk.

Milk No.	Time of pumping.	Amount per 100 cc. of milk.					Remarks.
		Total non-protein nitrogen.	Urea nitrogen.	Amino nitrogen.	Preformed creatinine.	Creatine.	
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	
186	6.00 a.m.	25	11.6	—	1.2	1.9	Wet-nurse B. R. W., age 23 years; weight 145 lbs.
187	9.00 "	24	9.0	—	1.2	2.1	
188	12.00 m.	25	10.4	8.1	1.3	2.7	
189	3.00 p.m.	26	11.0	—	1.2	2.1	Baby, age 8 wks.; weight 12 lbs., 15 oz.; gaining.
190	6.00 "	26	12.3	8.6	1.4	1.9	
191	9.00 "	25	9.2	8.4	1.4	1.9	
192	6.00 a.m.	30	13.3	8.7	1.2	1.9	Wet-nurse M. N.; age 26 years; weight 150 lbs.
193	9.00 "	33	13.3	—	1.2	2.0	
194	12.00 m.	28	9.5	8.9	1.2	2.0	
195	3.00 p.m.	28	11.0	—	1.2	2.1	Baby, age 8 wks., 3 days; weight 10 lbs., 9 oz.; gaining.
196	6.00 "	28	11.3	8.3	1.1	2.2	
197	9.00 "	28	11.7	—	1.1	2.1	
198	6.00 a.m.	30	12.0	6.8	1.0	2.2	Wet-nurse M. N.; age 20 years; weight 104 lbs.
199	12.00 m.	29	12.5	8.3	1.0	2.2	
200	3.00 p.m.	29	13.3	8.3	1.2	2.1	
201	6.00 "	28	12.9	7.0	1.1	2.2	Baby, age 11 wks.; weight 10 lbs., 12 oz.; gaining.
202	9.00 "	27	14.7	6.8	1.2	1.9	
205	6.00 a.m.	29	14.3		1.1	2.1	Wet-nurse S. McG.; age 23 years; weight 122 lbs.
206	9.00 "	29	14.0	3.6	1.1	1.9	
207	12.00 m.	28	11.1	4.4	1.1	2.1	Baby, age 9 wks.; weight 8 lbs., 12 oz.; has begun to gain only in last 3 weeks; fed part breast and part bottle.
208	3.00 p.m.	27	9.5	5.2	1.2	1.9	
209	6.00 "	28	11.0	4.6	1.1	2.0	
210	9.00 "	32	15.3	—	1.3	1.9	
203	6.00 a.m.	22	8.0	4.2	1.1	1.9	Wet-nurse J. B.; age 22 years; weight 130 lbs.
204	12.00 m.	22	7.4	—	1.2	2.3	Baby, age 12 wks.; not gaining. Mother has insufficient food.
211	6.00 a.m.	25	8.1	3.8	1.2	2.1	Wet-nurse R. M.; age 23 years; weight 142 lbs.
212	9.00 "	26	9.0	3.8	1.1		Baby, age 31 wks.; weight 10 lbs., 12 oz.; fed recently on bottle only.
213	12.00 m.	25	10.0	3.7	1.3	2.0	
214	3.00 p.m.	25	8.3	5.2	1.2	2.0	
215	6.00 "	25	10.2	3.7	1.1	2.1	
216	9.00 "	25	10.3	3.3	1.2	2.1	

TABLE II.

Non-Protein Nitrogenous Constituents of Human Milk.

Mother No.	Amount per 100 cc. of milk.					
	Total non-protein nitrogen.	Urea nitrogen.	Amino nitrogen.	Preformed creatinine.	Creatine.	Uric acid.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
H.	37	13.0	—	1.1	2.7	—
L.	24	10.5	—	1.1	2.6	—
F.	27	14.0	—	1.3	2.6	—
224	26	12.0	4.58	1.1	3.9	2.2
H50	32	17.0	4.74	1.5	2.1	2.6
H51	27	13.0	8.46	—	—	1.7
H52	36	16.0	8.58	—	—	—
H53	30	14.0	—	1.3	—	4.4
H54	36	15.3	5.6	1.6	—	3.8
H55	30	14.0	—	—	—	2.3
H56	35	16.0	—	1.3	—	2.4
236, 7	37	12.1	8.5	1.6	2.2	2.7
338, 9	36	12.2	8.6	1.6	2.2	2.5
240, 1	36	13.8	8.6	1.3	2.4	2.7
145	30	12.4	4.7	—	—	—
147	29	12.6	—	—	—	—
149	27	11.7	4.5	—	—	—
162	25	12.0	—	—	—	—
156	31	9.3	—	—	—	—
161	27	8.3	—	—	—	—
164	26	12.0	—	—	—	—
R.	35	15.6	—	—	—	—
30	26	10.6	—	—	—	—
154	27	13.3	—	—	—	—
168	30	10.0	—	—	—	—
164	27	12.0	—	—	—	—
H1	29	12.0	—	—	—	—
H2b	28	13.0	5.6	—	—	—
H2a	29	14.0	5.1	—	—	—
H3b	29	12.0	3.0	—	—	—
H3a	27	12.0	3.1	—	—	—
H4	26	11.4	—	—	—	—
H5	32	13.6	4.5	—	—	—
H5	20	9.5	—	—	—	—
H6	22	11.0	4.9	—	—	—
H7	20	9.5	—	—	—	—
H8	24	11.0	—	—	—	—
H9	20	13.0	—	—	—	—
H10	26	11.3	—	—	—	—
74	29	15.6	—	—	—	—

TABLE III.

Non-Protein Nitrogen and Urea in Blood and Milk.

Mother No.	Amount per 100 cc. of milk.		Amount per 100 cc. of blood.	
	Total non-protein nitrogen.	Urea nitrogen.	Total non-protein nitrogen.	Urea nitrogen.
	mg.	*mg.*	*mg.*	*mg.*
F.	29	12.2	25	11
S.	28	13.7	28	14
C.	29	12.3	25	12
M.	32	13.6	29	14
P.	20	9.5	27	14
M.	22	11	26	13
B.	24	11	26	12
T.	26	11.3	27	14
C.	26	11.4	25	12
103	25	11	26	13
149	27	12	31	13
163	39	17	40	20
156, 7	31	16	37	18
160	27	14	42	20
158	40	17	42	21
245, 6	28	13	32	16
55	30	14	30	13
H50	32	15	29	14
224	26	12	35	17
H56	36	16	35	16

EFFICIENCY OF OAT PROTEIN IN ADULT HUMAN NUTRITION.*

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In previous experiments in this laboratory nitrogen equilibrium was maintained in a subject weighing 54 to 55 kilos on a daily diet furnishing 2,100 calories with 30 gm. of protein of which 88 per cent was from maize (corn-meal), 10 per cent from milk, and 2 per cent from apple.[1]

The present experiments were designed to study the efficiency of the proteins of oatmeal in adult human nutrition when similarly supplemented by small amounts of milk, and to a less extent the efficiency of the oat protein without the milk supplement. The subjects were two healthy young women, Subject O weighing 54 kilos and Subject P weighing 67 kilos. The former (Subject O) had served as subject in the corn-meal (maize protein) experiments above mentioned and the arrangements and conditions surrounding the present experiments were similar to those described in the paper dealing with the efficiency of the maize proteins.[1]

The food consisted essentially of oatmeal cooked with starch and butter in thin hard "scones" and eaten with apple and sugar, in some experiments with, in others without, a small amount of milk. The diet was planned to parallel that of the previous investigation[1] as closely as possible so as to facilitate and make accurate the comparison of the nutritive efficiency of the protein mixture of oats with that of maize in the maintenance metabolism of the human adult.

* The authors desire to express their indebtedness to the Department of Social Welfare, New York Association for Improving the Condition of the Poor, which shared in the expenses of this investigation by a grant from the research fund established by Mrs. Elizabeth Milbank Anderson.

[1] Sherman, H. C., and Winters, J. C., *J. Biol. Chem.*, 1918, xxxv, 307.

In planning the diet of Subject O, we first analyzed the oatmeal, and thus found the amount required to furnish the same quantity of protein as was contained in the diet of the same subject in the maize protein experiment referred to above, which had been carried out only about 1 month previous to the oatmeal experiments here described. The quantity of oatmeal, thus required to furnish the same amount of nitrogen as 300 gm. of corn-meal, was 168 gm. This quantity of oatmeal was therefore consumed daily by this subject. The quantities of milk, butter fat, and apple used were the same as in the maize experiment but it was found necessary to increase the amount of sugar by 15 gm. and to add 95 gm. of corn-starch in order to make the total calories the same with the oatmeal as with the corn-meal. The total nitrogen intake of Subject O in the main experiment (Experiment 2) was then (after the introduction of the corn-starch) 5.0 gm. per day of which 85 per cent was from oatmeal, 10 per cent from milk, 2 per cent from corn-starch, and 3 per cent from apple.

The quantity of oatmeal was increased for Subject P to make the nitrogen intake proportionate to her greater body weight, and larger amounts of apple, butter fat, and corn-starch were consumed in order to afford a sufficient energy value. The total nitrogen intake in the main experiment with Subject P (Experiment 4) was 5.8 gm. per day of which 87 per cent was from oatmeal, 8 per cent from milk, 2 per cent from corn-starch, and 3 per cent from apple.

The experimental diets of both subjects furnished about 38 calories per kilo of body weight per day, except in Experiment 5 when the food intake of Subject P was lower as will be described later. That this allowance of 38 calories per kilo per day was well adjusted to the muscular activity of the subjects is indicated both by previous experience in similar cases and by the relative constancy of the body weights of both subjects during the experiments.

The experiments with Subject O covered 16 consecutive days of which the first 4 days (Experiment 1) constituted essentially a preparatory period without milk in order that the main period (Experiment 2) might be strictly comparable with the corresponding experiment in the maize protein investigation.

The experiments with Subject P covered 28 consecutive days. The first 12 days constituted Experiment 3 on a diet of oatmeal, corn-starch, sugar, butter, and apple only; the second 12 days, the main experiment (Experiment 4), in which 100 gm. of milk were added to the daily ration, the butter being diminished by 7 gm. in order that the total calorie intake should not be changed.

In the main experiments of 12 days each the two subjects, receiving only sufficient calories to meet the energy requirement and but 0.57 or 0.55 gm. of protein per kilo of body weight, showed respectively nitrogen balances of ±0.0 and +0.2 gm. of nitrogen per day. The nitrogen balance in the directly comparable experiment with maize protein made 1 month previously by Subject O was ±0.1 gm. of nitrogen per day. The three results may be regarded as practically identical.

Moreover the negative balances of Subject P during 12 days on the oat diet without milk and of Subject O during 8 days on the comparable maize diet without milk were practically alike averaging −0.7 and −0.6 gm. of nitrogen per day respectively.

It is evident therefore that in the maintenance metabolism of adults as shown by nitrogen balance experiments, the proteins of oats and maize are of virtually equal nutritive efficiency; and this is true whether the proteins in question constitute practically the sole nitrogenous food or are supplemented by a constant small amount of milk protein. That nitrogen equilibrium was maintained with such low protein intake in the latter experiments, and was so closely approached in the former, shows that the proteins of both maize and oatmeal were very efficiently utilized in the maintenance metabolism of these healthy adults.

When these experiments were begun the chief available evidence from the feeding of laboratory animals which seemed to bear directly upon the value of oat protein in rations similar to the food of man, was that of McCollum, Simmonds, and Pitz[2] who concluded that "the oat kernel seems to contain proteins of a poorer quality than either the maize or wheat kernel" and that "casein does not appear to supplement the proteins of the oat kernel in a very satisfactory manner."[3] In a later paper

[2] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1917, xxix, 341.

[3] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1917, xxix, 342.

McCollum and Simmonds give results which indicated a relatively higher value of oat proteins for maintenance.[4] The experiments of Osborne and Mendel[5] published in June, 1918, (while our work was in progress) appeared favorable to the nutritive efficiency of oat protein, especially when supplemented by casein, and in his summary published last October McCollum indicates[6] that the proteins of oats are superior to those of maize and wheat for the maintenance of body weight in the adult rat. From the data published by McCollum we are unable to judge whether the amounts of food consumed by the rats were sufficiently uniform to make the results directly comparable with those of our experiments in which human subjects ate exactly predetermined amounts of food thus insuring uniform intakes of the proteins to be compared as well as uniform energy values in the daily intake of total food.

Our experiments indicate that for the purposes of practical dietetics equal weights of oat and maize proteins may be regarded as essentially equal in value, and that even the minimum amount of milk which can possibly be regarded as permissible in the light of our present knowledge of nutrition, will apparently so supplement the proteins of either the maize or oat kernel as to make them function with an efficiency comparable with that of the average protein of mixed diet in the maintenance metabolism of man.

EXPERIMENTAL.

The 16 experimental days with Subject O were divided into two experiments: Experiment 1 consisting of a single 4 day period which sufficed in this case because the nitrogen balance of the same subject had been studied under parallel conditions a few weeks previously and it had been found that with such a diet she came quickly to a fairly constant level of nitrogen output; and Experiment 2 in three periods of 4 days each, this 12 day trial of uniform diet constituting the main experiment with Subject O.

The 28 experimental days with Subject P were divided as follows: Experiment 3 of 12 days duration subdivided into three

[4] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1917, xxxii, 347.
[5] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 521.
[6] McCollum, E. V., Newer knowledge of nutrition, New York, 1918, 75.

4 day periods; Experiment 4 also of 12 day duration and similarly subdivided; and a final brief Experiment 5 of 4 days.

The food consumed daily during each of the five experiments is shown in Table I.

The oatmeal was prepared in a manner similar to that employed in the case of the maize protein experiments,[1] being mixed in the case of Subject O with the 95 gm. of corn-starch and added together with 40 gm. of butter fat and 5 gm. of salt to 440 gm. of hot water. This was cooked directly over the flame until a thick mush was obtained which was spread on pans in sheets $\frac{1}{4}$ to $\frac{3}{8}$ of an inch thick and put in a Freas electric oven for a period of 2 hours and, when done, was quite dry and crisp throughout but not brown. Analysis of the finished product of control baking tests showed that no nitrogen was lost during the cooking. Subject P used 200 gm. of oatmeal, 100 gm. of corn-starch, 60 gm. of butter, 10 gm. of salt, 20 gm. of sugar, and 500 gm. of water.

The larger quantities of food consumed by Subject P in Experiments 3 and 4 as compared with Subject O in Experiments 1 and 2 were in order that the intake of protein and calories might be proportional to her larger body weight. Since this subject felt that her total food was somewhat more than sufficient and the body weight showed a slight tendency to rise, it was thought well to include a final period of 4 days (Experiment 5) in which the total calories were reduced about 10 per cent by withdrawing 30 gm. of butter fat from the daily diet without any other change in the ration. This resulted promptly in moderate losses of body weight and body nitrogen but tended to check the loss of body calcium. From this it would appear that in the main experiments the energy value of the diet was closely adjusted to the energy requirement of the subject, but that the intake of fat, while not unusually high, was slightly greater than would have been most advantageous to the utilization of a restricted intake of calcium.

The full data of intake and output are shown in Tables I to V. Only distilled water was used. In some cases this was sweetened with a part of the sugar of the ration and flavored with not over 1 gm. per day of pure citric acid.

Although the experiments were made in June the weather was

unusually cool and there was no reason to fear any significant loss of nitrogen through the perspiration.

In Experiment 1 (preliminary) of Subject O, 95 per cent of the protein of the diet was furnished by oatmeal, while the other 5 per cent was furnished by apple and corn-starch. In Experiment 2, 85 per cent of the protein of the diet was oat protein, 2 per cent was furnished by corn-starch, 3 per cent by apple, and 10 per cent by milk. Table IV shows an average nitrogen balance for this experiment of ± 0. Under the same conditions with the maize protein Subject O obtained a nitrogen balance of $+0.1$ gm. per day. These results are practically identical.

In Experiment 3 (Subject P), 95 per cent of the protein of the diet was oat protein, 3 per cent was furnished by apple, and 2 per cent by corn-starch (Same as Experiment 1, Subject O). In Experiment 4, Subject P, 87 per cent of the protein was furnished by oatmeal, 2 per cent by corn-starch, 3 per cent by apple, and 8 per cent by milk. Table IV shows that the nitrogen balance for the experiment without the milk protein was -0.7 gm. per day which changes to $+0.2$ when milk sufficient to furnish 8 per cent of the protein is added to the diet. This accords with the results of Osborne and Mendel in which casein efficiently supplemented the protein of the oat kernel.

TABLE I.

Kinds and Amounts of Food Eaten with Daily Intake of Calories, Protein, and Calcium.

Kind of food.	Subject O; weight 54 kg.		Subject P; weight 67 kg.		
	Experiment 1.	Experiment 2.	Experiment 3.	Experiment 4.	Experiment 5.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
Oatmeal	168	168	200	200	200
Corn-starch	95	95	100	100	100
Apple	300	300	375	375	375
Butter	70	70	95	88	58
Sugar	50	50	60	60	60
Milk	—	100	—	100	100
Calories	2,031	2,097	2,492	2,498	2,222
Protein	28	31	33	36	36
Nitrogen	4.5	5.0	5.3	5.8	5.8
Calcium	0.19	0.31	0.24	0.36	0.36

TABLE II.

Amounts and Proportions of Nitrogen and Calcium from Different Foods in Each Experiment.

	Subject O; weight 54 kg.				Subject P; weight 67 kg.					
Experiment No.	1		2		3		4		5	
Nitrogen.										
	gm.	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*
Total	4.49		4.99		5.32		5.81		5.81	
Oatmeal	4.25	95	4.25	85	5.06	95	5.06	87	5.06	87
Corn-starch	0.11	2	0.11	2	0.11	2	0.11	2	0.11	2
Apple	0.13	3	0.13	3	0.15	3	0.15	3	0.15	3
Milk	—	—	0.50	10	—	—	0.49	8	0.49	8
Calcium.										
Total	0.19		0.31		0.24		0.35		0.35	
Oatmeal	0.15	79	0.15	48	0.18	77	0.18	51	0.18	51
Corn-starch	0.01	5	0.01	3	0.01	4	0.01	3	0.01	3
Apple	0.03	16	0.03	10	0.04	19	0.04	12	0.04	12
Milk			0.12	39			0.12	34	0.12	34

TABLE III.

Daily Output of Nitrogen and Calcium in Experiments 1 and 2; Subject O.

Experimental period.	Date.	Body weight.	Nitrogen.			Calcium.		
			Urine.	Feces.	Total.	Urine.	Feces.	Total.
Experiment 1.								
	1918	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Period I.	May 31	54.4	6.3	1.0	7.3	0.04	0.26	0.30
	June 1		4.1	1.0	5.1	0.03	0.27	0.30
	" 2		3.5	1.0	4.5	0.06	0.26	0.32
	" 3		3.3	1.0	4.3	0.05	0.27	0.32
Experiment 2.								
Period I.	June 4	53.7	3.7	1.1	4.8	0.09	0.35	0.44
	" 5		4.1	1.1	5.2	0.10	0.35	0.45
	" 6		3.7	1.1	4.8	0.09	0.36	0.45
	" 7		3.7	1.1	4.8	0.10	0.36	0.46
" II.	" 8	54.4	4.2	1.0	5.2	0.17	0.27	0.44
	" 9		3.0	1.0	4.0	0.13	0.27	0.40
	" 10		3.7	1.0	4.7	0.13	0.27	0.40
	" 11		4.1	1.0	5.1	0.13	0.27	0.40
" III.	" 12	53.6	4.2	1.0	5.2	0.12	0.31	0.43
	" 13		4.5	1.0	5.5	0.14	0.31	0.45
	" 14		4.0	1.0	5.0	0.09	0.31	0.40
	" 15		4.2	1.0	5.2	0.08	0.31	0.39

TABLE IV.

Daily Output of Nitrogen and Calcium in Experiments 3, 4, and 5; Subject P.

Experimental period.	Date.	Body weight.	Nitrogen.			Calcium.		
			Urine.	Feces.	Total.	Urine.	Feces.	Total.
Experiment 3.								
	1918	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Period I.	May 31	67.3	5.8	1.1	6.9	0.04	0.39	0.43
	June 1		4.7	1.1	5.8	0.07	0.39	0.46
	" 2		4.1	1.1	5.4*	0.07	0.39	0.46†
	" 3		3.9	1.1	5.2*	0.07	0.39	0.46†
" II.	" 4	66.2	4.9	1.5	6.6*	0.12	0.31	0.43†
	" 5		4.4	1.5	6.1*	0.11	0.31	0.42†
	" 6		4.9	1.5	6.4	0.15	0.31	0.46
	" 7		4.9	1.5	6.4	0.14	0.31	0.45
" III.	" 8	67.7	4.5	1.3	5.8	0.09	0.28	0.37
	" 9		4.4	1.3	5.7	0.09	0.28	0.37
	" 10		4.8	1.3	6.1	0.12	0.28	0.40
	" 11		4.4	1.3	5.7	0.12	0.28	0.40
Experiment 4.								
Period I.	June 12	68.5	4.7	1.4	6.1	0.17	0.37	0.54
	" 13		4.1	1.4	5.5	0.14	0.38	0.52
	" 14		4.5	1.4	5.9	0.18	0.37	0.55
	" 15		4.0	1.4	5.4	0.19	0.38	0.57
" II.	" 16	68.8	3.6	1.3	4.9	0.16	0.35	0.51
	" 17		3.9	1.3	5.2	0.13	0.35	0.48
	" 18		4.1	1.3	5.4	0.14	0.35	0.49
	" 19		3.9	1.3	5.2	0.13	0.35	0.48
" III.	" 20	68.2	4.2	1.8	6.0	0.14	0.43	0.57
	" 21		4.0	1.8	5.8	0.15	0.43	0.58
	" 22		4.3	1.8	6.1	0.17	0.43	0.60
	" 23		3.3	1.8	5.1	0.15	0.43	0.58
Experiment 5.								
Period I.	June 24	67.6	4.0	1.3	5.3	0.14	0.35	0.49
	" 25		4.9	1.3	6.2	0.12	0.35	0.47
	" 26		5.0	1.3	6.3	0.14	0.35	0.49
	" 27	66.1	5.5	1.3	6.8	0.14	0.35	0.49

* Including 0.2 gm. of nitrogen for each of these 4 days from the menstrual flow.

† Including 0.005 gm. of calcium or each of these 4 days from the menstrual flow.

TABLE V.

Summary of Daily Intake and Output of Nitrogen and Calcium in Experiments 1, 2, 3, 4, and 5.

Experiment No.	Period.	Body weight.	Nitrogen.			Calcium.		
			Intake.	Output.	Balance.	Intake.	Output.	Balance.
			Subject O.					
		kg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	I	54	4.5	5.3	−0.8	0.19	0.31	−0.12
2	I	53.7	5.0	5.0	±0.0	0.31	0.45	−0.14
	II	54.4	5.0	4.8	+0.2	0.31	0.41	−0.10
	III	53.6	5.0	5.3	−0.3	0.31	0.42	−0.11
Average		54	5.0	5.0	±0.0	0.31	0.43	−0.12
			Subject P.					
3	I	67.3	5.3	5.8	−0.5	0.24	0.45	−0.21
	II	66.2	5.3	6.4	−1.1	0.24	0.44	−0.20
	III	67.7	5.3	5.8	−0.5	0.24	0.38	−0.14
Average		67	5.3	6.0	−0.7	0.24	0.42	−0.18
4	I	68.5	5.8	5.8	±0.0	0.35	0.54	−0.19
	II	68.8	5.8	5.2	+0.6	0.35	0.49	−0.14
	III	68.2	5.8	5.8	±0.0	0.35	0.58	−0.23
Average		68.5	5.8	5.6	+0.2	0.35	0.53	−0.18
5		67	5.8	6.2	−0.4	0.35	0.48	−0.13

VITAMINE STUDIES.

IV. ANTINEURITIC PROPERTIES OF CERTAIN PHYSIOLOGICAL STIMULANTS.*

PRELIMINARY PAPER.

BY R. ADAMS DUTCHER.

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(Received for publication, June 20, 1919.)

INTRODUCTION.

In previous publications, (1) I have attempted to show that avian polyneuritis is probably accompanied by a lowering of the oxidative power of the body tissues (as measured by catalase activity) and that the oxidative processes tend to return to normal upon the administration of an alcohol-water extract of wheat germ or corn pollen. It was likewise shown that a high carbohydrate diet hastened the onset of polyneuritic symptoms when the diet was deficient in regard to the water-soluble vitamine. While it was not possible to draw definite conclusions, it was suggested that one function of the water-soluble vitamine, of the type found in wheat embryo, might be that of stimulating the oxidative processes, thereby clearing the tissues of toxic substances which have accumulated due to incomplete or partial oxidation.

That there is some relationship between vitamine content of food materials and endocrine function there is little doubt, for McCarrison (2) has shown that vitamine deficiency (in pigeons) causes a progressive atrophy of many of the vital organs, including the ductless glands (with the exception of the adrenals which show a tendency to hypertrophy). It is not improbable that the improved condition noted in pigeons after the administration of water-soluble vitamine and other curative substances and the apparent increased metabolic rate noted in deficiency studies,

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when the lacking essential is supplied, are due to the increased activity of the glands of the alimentary tract and organs of internal secretion, with the result that food materials are more completely digested and assimilated and the stimulation of cell activity, due to hormone action, is increased. In this regard Uhlmann (3) found that vitamine extracts from various plant sources acted in a manner very similar to pilocarpine increasing gland secretion and, like pilocarpine, the action of these extracts was antagonized by atropine.

While I have approached the problem from the standpoint of physiological action, others have attempted to answer the question by feeding compounds of known chemical structure, basing their work upon the chemical nature of substances isolated from vitamine extracts. Funk (4) believed that the curative substance, which he isolated from rice polishings and yeast, was chemically related to the pyrimidines or purines; consequently he fed several of these compounds achieving more or less success with allantoin, hydantoin, thymus nucleic acid, and cytidine. Williams (5) found that the hydroxyl pyridines possessed antineuritic properties which were lost on long standing or after undergoing certain chemical treatment. He believes this change to be tautomeric in nature and upon this hypothesis he has suggested that the instability of vitamines can be explained on a similar basis. Publishing with Seidell (6), the same author describes the isolation of a substance from yeast which is isomeric with adenine. This substance was also shown to exist in a curative and non-curative form. In a more recent paper Williams (7) concludes that the curative form of α-hydroxyl pyridine is, structurally, a pseudobetaine, and from this he postulates that the curative form of nicotinic acid (and possibly antineuritic vitamines) possesses similar chemical characteristics. Harden has questioned the view that α-hydroxyl pyridine cures or improves the condition of polyneuritic birds (8).

In order to obtain a clearer understanding of the mechanism of vitamine action, I have conducted some preliminary experiments with the view of attempting to find, eventually, chemical substances, whose physiological action and chemical constitution have been studied, which will bring about physiological response similar to that of the vitamine extracts. The data thus obtained may afford information which will assist us in studying further

the chemical nature of the water-soluble vitamine as well as to shed some light on the mechanism of vitamine action. Thus far the only substances which have been fed in this preliminary work are thyroxin, desiccated thyroid gland, pilocarpine hydrochloride, and tethelin. Experiments are now in progress to verify the present work with a larger number of birds and to extend the study to other chemical substances.

EXPERIMENTAL.

Experiments with Thyroxin.—Kendall (9) has isolated what he considers to be the thyroid hormone from the thyroid gland and submits data to show that the substance is "tri-hydro-tri-iodo-α-oxy-indol propionic acid." Through the generosity of Dr. Kendall, I have been able to use this substance in the preliminary work, feeding in all, five polyneuritic birds. Kendall has shown that thyroxin increases the rate of metabolism and suggests that the active chemical groups in this compound "are a necessary mechanism for the production of energy within the body (10)." He has also observed that the administration of thyroxin, like our vitamine extracts, tends to bring subnormal temperatures back to the normal.

Because of these apparent similarities, it was considered of interest to see if thyroxin would show a curative effect in avian polyneuritis. Polyneuritis was induced by feeding diets of polished rice or diets composed of polished rice which had been soaked in water, the water drained off, and the wet rice supplemented by the addition of 3 gm. of purified casein to each 100 gm. of dry rice. The casein was soaked and dried upon the rice surfaces after mixing thoroughly. With but two exceptions the pigeons on the casein-rice diet became polyneuritic earlier than those on ordinary polished rice, and it might be added that of the total number of birds started on the casein-rice diet at the beginning of the experiment but 50 per cent became polyneuritic; the remainder, having refused to eat, died of starvation. All the birds which were fed on polished rice became polyneuritic.

Pigeon 28 was found in the acute stage of the disease on the 33d day of the experiment and was given 6 mg. of thyroxin. On the following day the bird was found walking about the cage and all paralytic symptoms had disappeared. This bird had refused to eat its diet of rice for several days, with the result that it died of starvation on the 5th day after the administration of thyroxin. However, no polyneuritic symptoms were detected after the apparent relief 5 days before death. Pigeon 35 became polyneuritic on the 17th day of feeding and 1 mg. of thyroxin was injected subcutaneously. The bird grew steadily worse and 2 mg. more of thyroxin

were fed (by tube into crop) at 9 a.m. on the following day. At noon the pigeon was on its feet and attempting to eat, but the typical spasms returned at 3 p.m. At 3.45 p.m. the symptoms had disappeared and the bird grew steadily better and was apparently normal and very active for 11 days, at the end of which time the acute symptoms returned. This condition was relieved by the feeding of 1 mg. of thyroxin. After 2 days, in order to save the bird, inasmuch as it had become exceedingly emaciated and refused to eat, it was given a vitamine preparation. Pigeon 36 was so emaciated by the time the symptoms became evident that it died before thyroxin had time to show an effect. It should be noted here that thyroxin apparently requires 24 to 48 hours for its action to become evident,[1] in this respect it is unlike our vitamine extracts which, as a rule, show their effect in a very few hours.

Pigeon 43 lost all evidences of polyneuritis and lived for 5 days when given 10 mg. of thyroxin not so highly purified as that described above. Pigeon 45 died a few hours after receiving 1 mg. of thyroxin, due, apparently, to inanition.

Pigeons 33 and 50 recovered and then returned to the paralytic state when fed 200 mg. of desiccated thyroid gland. It is possible that we might have noted a greater effect if larger amounts had been fed.

Experiments with Pilocarpine.—Pigeon 41 was found in the typical paralyzed state in the morning and received 0.5 mg. of pilocarpine hydrochloride subcutaneously. At 1 p.m. of the same day the bird was found walking about the cage in normal condition, but rather weak. This bird finally died 3 days later, but no further symptoms of paralysis were evident.

Pigeon 44 did not respond to pilocarpine but died during the first attack of spasms.

Pigeon 49 became polyneuritic without great loss of body weight, as contrasted with the case of the birds just described. This pigeon was relieved with pilocarpine and remained normal and lively for 18 days, at the end of which time polyneuritis again appeared and although pilocarpine again relieved the paralysis, death occurred, probably from inanition.

Experiments with Tethelin.—Two pigeons were fed tethelin, a substance isolated by Robertson (11) from the anterior lobe of the pituitary body.

Pigeon 51 showed slight improvement when given 50 mg. of tethelin but died 5 days later.

Pigeon 52, on the other hand, was relieved with 70 mg. of tethelin and lived for 26 days after recovering from the paralysis.

The results of these experiments are given in Table I.

CONCLUSIONS AND SUMMARY.

The substances used, *i.e.* thyroxin, desiccated thyroid, pilocarpine, and tethelin, apparently produced definite relief in cer-

[1] Dr. Kendall informs me that a similar period of time usually elapses before physiological response is obtained in humans.

TABLE I.

Summarization of Data Obtained in Experiments Where Thyroxin, Desiccated Thyroid, Pilocarpine, and Tethelin Were Administered to Polyneuritic Pigeons.

Pigeon No.	Diet.	Time before becoming polyneuritic.	Original weight.	Weight at polyneuritic stage.	Final weight.	Body weight lost at time of		Substance tested.	Results.
						Polyneuritis.	Death.		
		days	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*		
28	Rice.	33	358	244		31.8		Thyroxin.	Positive improvement.
35	Casein-rice.	17	277	231.	179	16.6*	35.4†	"	" "
36	"	29	265	170	170	35.8	35.8	"	Died before thyroxin could show effect.
43	"	16	326	285	270	12.5	17.2	"	Positive improvement.
45	"	28	316	200	200	36.7	36.7	"	Died before thyroxin could show effect.
41	"	42	352	215	225	38.9	36.0	Pilocarpine.	Positive improvement.
44	"	42	305	140	140	54.1	54.1	"	Died, very much emaciated.
49	Rice.	43	275	225	200	18.2	27.3	"	Positive improvement.
51	"	51	364	271	250	25.5	31.3	Tethelin.	Slight "
52	"	49	359	242	179	32.6	50.0	"	Positive "
33	Casein-rice.	33	410	262	231	36.1	43.7	Desiccated thyroid.	Positive but temporary improvement.
50	Rice.	32	261	216	205‡	17.2	21.4‡	" "	Positive but temporary improvement.

*Represents the per cent loss in weight at the time of the first attack of polyneuritis. This bird was relieved twice.

†Represents the weight at which the diet was changed to wheat.

‡Pigeon killed for experimental purposes.

tain acute cases of avian polyneuritis. However, in none of the cases described did we obtain the almost immediate response that is usually observed when vitamine preparations are fed. This is rather to be expected inasmuch as the vitamine preparations contain, in addition to the so called vitamine, phosphorus, sulfur, and organic compounds which undoubtedly are needed in the repair and building of tissue. This view is substantiated by the fact that the very emaciated birds responded slowly, if at all, to the chemicals fed, while the less emaciated birds responded much more quickly, indicating that possibly the tissues contained a reserve supply of nutrients sufficient for immediate anabolic processes following the stimulatory action of the substances employed.

Pigeons have been relieved of the paralytic symptoms, in avian polyneuritis, by means of thyroxin, desiccated thyroid gland, pilocarpine hydrochloride, and tethelin. One pigeon was relieved for a period of 11 days by means of thyroxin, another lived for 18 days after being relieved by means of pilocarpine hydrochloride, and another lived for 26 days after the administration of tethelin. The work is being continued along the lines indicated by the above observations.

In conclusion, I wish to take this opportunity to thank Dr. E. C. Kendall for the thyroxin used in this work, and also Armour and Company of Chicago to whom I am indebted for the desiccated thyroid preparation.

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EPICHITOSAMINE AND EPICHITOSE.

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(Received for publication, June 30, 1919.)

Only two α-aminohexoses have been isolated in form of their simple salts: namely, chitosamine and chondrosamine. Xylohexosamine has been prepared only in form of its benzoyl derivative. These three sugars present the entire list of nitrogenous sugars prepared synthetically. The number of them found in nature is limited to two. In the present communication account is given of the synthesis of epichitosamine. This sugar and chitosamine will constitute the first known pair of epimeric hexosamines. Although interesting in itself the sugar was prepared principally for one definite reason. In a previous publication a comparison of the physical properties of three pair of epimeric acids was recorded. Two pair were prepared synthetically from the corresponding pentoses and there was no doubt as to their configuration save the allocation of the amino groups. In the third pair, the configuration of one had been established by Fischer. Its epimer has been recently prepared from chitosaminic acid by the action of pyridine.[1] Since there was no precedent as to the reaction of pyridine on hexosaminic acid, the purity of what was expected to be epichitosaminic acid had to be established experimentally. The fact that epichitosaminic acid formed a crystalline lactone under exactly the same conditions which bring about the formation of a benzal ester of chitosamine, and further, the fact that the lactone was converted into chitonic acid, seemed sufficient evidence in support of the assumed structure of epichitosaminic acid. Yet, additional proof seemed desirable, and because of this it was undertaken to convert the lactone into the corresponding sugar, with a view of reconverting the sugar into the α-aminohexosaminic acid.

[1] Levene, P. A., *J. Biol. Chem.*, 1918, xxxvi, 73.

Another object in preparing the sugar was the following. On action of nitrous acid on chitosamine and the corresponding aminohexonic acid, and on chondrosamine and its acid, in each instance, the sugar and the acid gave rise to epimeric anhydro acids. On the action of nitrous acid on dextroxylohexosaminic acid and on its lactone also two epimeric anhydro acids were obtained. Since lactones may be regarded as inner esters, and since Fischer has observed that amino-acids and the corresponding esters as a rule gave antipodal hydroxy acid, the observation on xylohexosaminic acid was in harmony with what was to be expected.

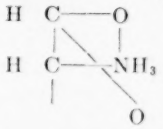
On the other hand, epichitosaminic acid and its lactone both gave, by the action of nitrous acid, chitonic acid. Instances where an amino-acid and its ester gave on deamination one and the same hydroxy acids were observed by Fischer. At first this author was inclined to assume that in such instances the Walden inversion did not occur; later he reconsidered his hypothesis and assumed that in these instances Walden inversion took place in both acid and ester. Both assumptions were more or less arbitrary and further experimental facts were desired for the correct interpretation of such observations. Because of these considerations it seemed desirable to test the action of nitrous acid not only on the lactone of epichitosaminic acid, but also on epichitosamine.

All attempts to carry out the original plan of the work proved futile. The sugar seemed to possess properties different from those observed on any other of the nitrogenous sugars.

First, the attempts to oxidize the sugar into the corresponding amino-acid failed. Both methods were employed; the one by means of bromine and the one by means of mercuric oxide. The temperatures and the duration of oxidation were varied, but the results were always negative.

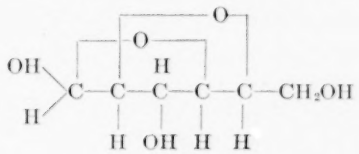
A second peculiarity of the sugar was its optical activity. It is the only sugar which showed no mutarotation. The substance was dissolved at 0°C. and the first reading of the solution taken immediately, but after 24 and after 48 hours no change in the initial reading was obtained. It seemed possible to explain this peculiarity by the assumption that this sugar in distinction

from the other ones had the betaine structure assumed by Irvine[2] for other amino sugars.



If such a distinction between the individual sugars were a reality one should have expected that they would react differently toward nitrous acid. However, it was found that all known amino sugars gave off their nitrogen on treatment with nitrous acid in about 5 minutes.

The third peculiarity of the sugar lay in its behavior towards mercuric oxide. While other sugars under this treatment were transformed into the corresponding amino-acids, epiglucosamine formed under these conditions epichitose. The only pleasant surprise in the work on the sugar was the fact that epichitose, unlike chitose, appeared in beautiful crystalline form. Similar to epichitosamine, epichitose showed no mutarotation. Epichitose may be represented graphically as follows:



A fourth peculiarity of the sugar was found in its behavior towards nitrous acid. Previous to this, six hexosaminic acids, two lactones, and two hexosamines have been acted upon by nitrous acid. In every instance the resulting substance was a ring compound, owing to the dehydration following the deamination. On treatment of epichitosamine with nitrous acid, saccharic acid was obtained instead of anhydrosaccharic acid, as one would have expected. The saccharic acid was identified by the rotation of its acid potassium salt. The acid potassium salt of anhydrosaccharic acid crystallizes with one molecule of water of crystallization, and because of this the two salts are not

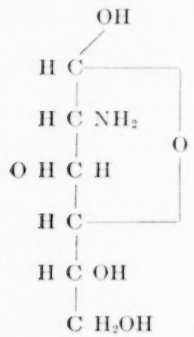
[2] Irvine, J. C., and Hynd, A., *J. Chem. Soc.*, 1912, ci, 1136.

analytically distinguishable. Fortunately they differ in their optical rotation. Also, the optical rotation of the calcium salt obtained on oxidation of epichitosamine showed the rotation of calcium salt of saccharic acid. However, the calcium salt was obtained in small quantities and not analytically pure, but the evidence of the acid potassium salt is sufficient to establish the identity of the substance.

The fact that on deamination of the sugar saccharic acid was formed, whereas on the same treatment of the epichitosaminic acid and its lactone the anhydromannonic acid was formed is very important in connection with that phase of the discussion on Walden inversion, which has been referred to earlier in this paper; namely, when an amino-acid and the corresponding ester give rise to the same hydroxy acid then both theories are admissible, either that the inversion does take place in both of them, or in neither of them, and that the choice between the two for the present is arbitrary.

As regards the structure of the epichitosamine, mention must be made of the evidence in favor of its being an α-amino sugar. It was found that the sugar on treatment with phenylhydrazine in the usual way gives rise to glucosazone, and on deamination of the lactone of epichitosaminic acid, α-α_1-anhydromannonic acid is formed.

The graphic representation of the structure of epichitosamine can therefore be represented in one of the following ways.



EXPERIMENTAL.

The reduction of the lactone into the sugar was carried out in the same manner as described in previous communications. Lots of 10.0 gm. of lactone hydrochloride were taken up in 60.0 cc. of water and reduced with 125.0 gm. of a 2 per cent sodium amalgam. The reaction of the solution was kept on the acid side by adding at short intervals small quantities of hydrochloric acid.

The filtrate from the mercury is concentrated at diminished pressure and at room temperature. Care has to be taken to carry out the concentration in such a manner that the concentrate remains colorless. The sodium chloride is separated by fractional precipitation first with methyl alcohol and subsequently with ethyl alcohol. After the product was obtained in such a state of purity that on ignition no ash was visible, the sugar was recrystallized once or twice out of water.

The analysis of the hydrochloride gave the following results. 0.1042 gm. of the substance gave 0.1298 gm. of CO_2 and 0.0614 gm. of H_2O. 0.010 gm. of the substance gave in Van Slyke's micro-apparatus 1.18 cc. of nitrogen gas at $T° = 21°$C. and $P = 765$.

0.020 gm. of the substance gave in Van Slyke's micro-apparatus in 5 minutes 2.43 cc. of nitrogen gas at $T° = 25°$ and $P = 764$.

The melting point of the substance was, M. P. = 187°C. (corrected).

	Calculated for $C_6H_{12}NO_5HCl$.	Found. I-328½	II-375½	III-111½
C	33.40		33.97	
H	6.54		6.61	
N	6.51	6.73		6.78
Cl	16.45			

The rotation of the substance in 5 per cent HCl solution was

$$[\alpha]_D^{20} = \frac{-0.47 \times 100}{1 \times 10} = -4.7 \qquad \frac{-0.47 \times 100}{1 \times 10} = -4.7$$

Initial. Equilibrium.

Preparation of the Osazone.

4.6 gm. of the sugar hydrochloride were taken up in 200 cc. of water, the solution was neutralized with sodium acetate, and a solution of 8.0 gm. of phenylhydrazine in 5 cc. of glacial acetic

acid were added and the entire solution allowed to stand on the boiling water bath for 1 hour. An osazone formed which was recrystallized from methyl alcohol. M. P. at rapid heating was 205°C. It had the following composition. 0.100 gm. of the substance gave 0.2202 gm. of CO_2 and 0.580 gm. of H_2O.

	Calculated for $C_{18}H_{24}N_4O_4$.	Found.
C	60.33	60.05
H	6.14	6.49

The rotation of the substance was, when 0.100 gm. was dissolved in 10 cc. of Neuberg's Pyridin and alcohol mixture: Initial −0.31, Equilibrium −0.15 which is in accord with the rotation found for glucosazone.

Oxidation of Epichitosamine with Nitric Acid.

Efforts to obtain the monocarboxylic acid failed, hence it was attempted to oxidize the sugar to the corresponding dicarboxylic acid. The conditions which led to a successful preparation of the substance are as follows. 10.0 gm. of the hydrochloride were dissolved in 100 cc. of water and to the solution were added 15.0 gm. of sodium nitrite and a few drops of concentrated hydrochloric acid. The flask was placed in an ice water bath and allowed to stand for 6 hours. At the end of that time the reaction product was placed on a boiling water bath for 3 minutes, filtered, and from the filtrate the excess of silver removed by means of hydrogen sulfide. The filtrate was concentrated to a volume of 35 cc. To this solution 35 cc. of concentrated nitric acid were added and the solution was rapidly evaporated on two large clock glasses. The residue was then dissolved by adding 5 cc. of water to each clock glass and again rapidly evaporated to dryness.

The residue was finally dissolved and converted into the calcium salt in the usual way. This salt did not analyze correctly for the expected dicarboxylic acid. However, the optical rotation of the substance differed from either anhydrosaccharic, or anhydromannosaccharic, but closely approached that of saccharic acid.

The composition of the substance (No. $303\frac{1}{1}\frac{8}{9}$) was the following. 0.1042 gm. of the substance gave 0.1070 gm. of CO_2, 0.0253 gm. of H_2O, and 0.0270 gm. of CaO.

	$C_6H_8O_8Ca$ calculated.	Found.
C	29.03	28.00
H	3.28	2.53
Ca	22.58	25.91

The rotation was

Initial. Equilibrium.

$$[\alpha]_D^{25} = \frac{+0.10 \times 100}{1 \times 2} = +5.0 \qquad [\alpha]_D^{25} = \frac{+0.20 \times 100}{1 \times 2} = +10.0$$

Calcium salt of:

Anhydromannosaccharic.

Initial. Equilibrium.

$$[\alpha]_D^{25} = \frac{+0.56 \times 100}{1 \times 2} = +28.0 \qquad [\alpha]_D^{25} = \frac{+0.55 \times 100}{1 \times 2} = 27.5$$

Anhydrosaccharic.

$$[\alpha]_D^{25} = \frac{+0.55 \times 200}{1 \times 2} = +27.5 \qquad [\alpha]_D^{25} = \frac{+0.58 \times 100}{1 \times 2} = 29.0$$

Saccharic acid.

$$[\alpha]_D^{25} = \frac{+0.09 \times 100}{1 \times 2} = +4.5 \qquad [\alpha]_D^{25} = \frac{+0.22 \times 100}{1 \times 2} = +11.0$$

It was then concluded to prepare the acid potassium salt. For that purpose 20.0 gm. of epichitosamine chlorohydrate were oxidized in two lots of 10.0 gm. each in exactly the same manner as in the previous experiment. The product of oxidation of each lot was taken up in 8 cc. of water, the solution was rendered alkaline by means of a 50 per cent solution of potassium hydrate, and then rendered acid by means of acetic acid. Methyl alcohol was added dropwise until slightly opalescent. The solution was then allowed to stand over night in the refrigerator. During that time a crystalline sediment formed. The substance was recrystallized once out of dilute alcohol, and once out of water containing a few drops of acetic acid. The yield of the crude product was 1.9 gm.

The substance had the following composition. 0.1000 gm. of the substance gave 0.0352 gm. of K_2SO_4.

	Calculated for $C_6H_9O_8K$. *per cent*	Found. *per cent*
K	15.70	15.81

The rotation of the substance in a 5 per cent solution of KOH was

$$[\alpha]_D^{25} = \frac{0.10 \times 100}{1 \times 2} = +5.0$$

Under the same condition a sample of the acid potassium salt obtained on oxidation of glucose possessed the following rotation.

$$[\alpha]_D^{25} = \frac{0.10 \times 100}{1 \times 2} = +5.0$$

A sample of the K salt of anhydrosaccharic acid showed the following rotation.

$$[\alpha]_D^{25} = \frac{+1.35 \times 100}{1 \times 2} = +67.5$$

Hence it is evident that the substance was the acid potassium salt of saccharic acid.

Epichitose.

15.0 gm. of ash-free epichitosamine hydrochloride were taken up in 250 cc. of water and 80.0 gm. of mercuric oxide, and heated on a boiling water bath for 30 minutes at the end of which time the greater part of oxide turned from orange to gray. Through the filtrate from the oxide hydrogen sulfide gas was passed and the filtrate from the sulfide was concentrated to a very thick syrup. This was taken up with a little methyl alcohol. On standing over night a crystalline deposit formed. It was nitrogen-free, it reduced Fehling's solution on heating, and possessed a melting point, M. P. = 240 (corrected), with decomposition.

The analysis of the substance gave the following results. 0.1031 gm. gave 0.1670 gm. of CO_2 and 0.0570 gm. of H_2O.

	Calculated. *per cent*	Found. *per cent*
C	44.44	44.18
H	6.17	6.18

The optical rotation of the substance was

Initial. $[\alpha]_D^{25} = \frac{-1.92 \times 100}{1 \times 2} = -96.0$ Equilibrium. $[\alpha]_D^{25} = \frac{-1.92 \times 100}{1 \times 2} = -96.0.$

CYTIDINE PHOSPHORIC ACID.

By P. A. LEVENE.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 30, 1919.)

The theoretical importance attached to the nucleotides obtained on acid hydrolysis of yeast nucleic acid has been discussed in a previous publication. It was there pointed out that the product of the acid hydrolysis was a mixture of two mononucleotides,[1] namely, of uridine phosphoric and of cytidine phosphoric acids. The former had been obtained in a crystalline form as a barium salt, the latter was prepared also as a barium salt. The barium salt of cytidine phosphoric acid possessed greater solubility in water, and had a tendency to settle out of a very concentrated solution in the form of a granular precipitate which consists of microscopic granules. On recrystallization it formed a deposit of irregular plates. The precipitate of barium uridine phosphate, when it settles out rapidly from its aqueous solution, not infrequently has a similar appearance. The difference in the behavior of the two salts is that barium uridine phosphate on recrystallization settles out in form of prismatic needles while the salt of cytidine phosphoric acid on recrystallization appears in form of microscopic granules.

EXPERIMENTAL.

The conditions of hydrolysis and of preparation of the crude brucine salts were given in the communication on the uridine phosphoric acid. The purification of the brucine salt of cytidine phosphoric acid was perfected since the date of the older publication. The process as carried out at present is as follows.

[1] Levene, P. A., *Proc. Soc. Exp. Biol. and Med.*, 1917, xv, 21; *J. Biol. Chem.*, 1918, xxxiii, 229.

The mixed brucine salt is dissolved in boiling 35 per cent alcohol in the proportion of 15.0 liters of alcohol to 100.0 gm. of the salts. The solution is allowed to cool and is kept at 10°C. for 24 hours. A crystalline deposit of brucine salts is formed, which is separated by filtration and again dissolved in 35 per cent alcohol in the same proportions as above. The process is repeated once more. The mother liquors of the three crystallizations are combined and concentrated under diminished pressure to a small volume. A crystalline deposit forms in the distilling flask. To complete the crystallization the contents of the flasks are allowed to stand over night at 10°C.

This product consists chiefly of the brucine salt of cytidine phosphoric acid, but contains a small amount of uridine phosphoric acid. The greater part of the latter is removed in the following way. The material is taken up in methyl alcohol in the proportion of 25.0 gm. of the brucine salts to 1 liter of alcohol and the alcohol is brought to a boil on the water bath. The greater part of the brucine salt of uridine phosphoric acid remains insoluble. The extract contains the salt of cytidine phosphoric acid. A part of it settles out on cooling the solution; the remainder crystallizes after the mother liquor is concentrated under diminished pressure to small volume.

The following analytical data of the three fractions represent the results of one of many similar experiments.

I.—Part insoluble in hot methyl alcohol.

0.200 gm. gave 13.8 cc. of N at $T° = 30°C$. $P = 757$ mm. N = 7.74 per cent.

II.—Part crystallized on cooling of hot extract.

0.200 gm. gave 15.6 cc. of N at $T° = 30°C$. $P = 757$ mm. N = 8.75 per cent.

III.—Part crystallized on concentration of mother liquor.

0.200 gm. gave 14.4 cc. of N at $T° = 14°C$. $P = 759$ mm. N = 8.27 per cent.

The theory for the brucine salt of uridine phosphoric acid $C_{55}H_{63}N_6PO_7 + 7\ H_2O$ requires N = 6.79 per cent.

The theory for the brucine salt of cytidine phosphoric acid $C_{53}H_{64}N_7PO_6 + 7\ HO_2$ requires N = 7.93 per cent.

Conversion of the Brucine Salts into Barium Salts.

The old process of this conversion has been modified, since it was found to be associated with considerable loss. The brucine salts are dissolved in boiling 35 per cent alcohol, to the solution an excess of ammonia is added, and the ammoniacal solution is concentrated under diminished pressure until free brucine begins to crystallize.

The brucine is removed by filtration, the mother liquor is again rendered strongly ammoniacal, and concentrated until brucine begins to crystallize. The operation was repeated as often as required, so that finally on concentration no perceptible quantity of brucine became evident. The brucine which separates out on concentration of the ammoniacal solution contains some of the brucine salts of nucleotides, and it is advisable to suspend them in hot water, treat the suspension with an excess of ammonia, filter them from free brucine, and to concentrate the mother liquor as above.

The next step in the process consists in obtaining the ammonium salts of the nucleotides. For this purpose they are precipitated out of their concentrated solution by absolute alcohol, by acetone, or by a mixture of equal parts of acetone and alcohol. The choice of the reagent depends upon the solubility of a given nucleotide, and if the solubility is not known a test should be made on a small sample of the solution of the ammonium salts. Thus the ammonium salt of uridine phosphoric acid is precipitated readily by means of absolute alcohol. The salt of cytidine phosphoric acid is precipitated more conveniently by a mixture of equal parts of alcohol and acetone. The yield of ammonium salts from the brucine salts is practically theoretical.

For conversion into the barium salt the ammonium salt of the nucleotide is dissolved in water and a slight excess of the theoretical amount of aqueous solution barium hydroxide is added. The solution is then evaporated at room temperature under diminished pressure nearly to dryness. This operation is repeated three times. The final residue is dissolved in a slight excess of dilute sulfuric acid bringing the volume to 1 liter for every 20 gm. of the ammonium salt. To this solution aqueous barium hydroxide is added until the solution turns very faintly pink with phenolphthalein. The product is then filtered and the filtrate

is concentrated under diminished pressure and at room temperature until the solution in the distilling flask turns slightly turbid. The contents of the flask are then allowed to stand at room temperature over night, filtered, and the filtrate is again concentrated as the first time. The operation is repeated as often as necessary.

The barium salts may be recrystallized by dissolving in water containing a slight excess of sulfuric acid. The solution is then treated in exactly the same manner as for the first precipitation of the barium salts.

The analysis of the barium salt of cytidine phosphoric acid gave the following results.

0.0842 gm. of substance used for Kjeldahl estimation required for neutralization 5.65 cc. of 0.1 N acid.

0.1316 gm. of substance gave 0.1144 gm. of CO_2, 0.0300 gm. of H_2O, and 0.0642 gm. of $Ba_2P_2O_7$.

0.1684 gm. of the substance gave 0.0388 gm. of $Mg_2P_2O_7$.

	Calculated for $C_9H_{12}N_3O_8PBa$.	Found.
C	23.56	23.71
H	2.64	2.55
N	9.16	9.39
P	6.77	6.43
Ba	29.96	29.36
$Ba_2P_2O_7$	48.97	48.79

The optical rotation of the air-dry substance was

$$[\alpha]_D^{20} = \frac{+0.56 \times 100}{1 \times 4} = +14.0.$$

Hydrolysis of the Substance.

8.0 gm. of the barium salt were suspended in 100 cc. of a 10 per cent solution of sulfuric acid and heated for 4 hours in a sealed tube at 125° in an oil bath. The product was filtered from barium sulfate. From the filtrate the phosphoric and sulfuric acids were removed by an excess of barium hydroxide and the excess of barium hydroxide removed quantitatively. The filtrate was then concentrated to a small volume and treated with an aqueous solution of picric acid. A light yellow precipitate formed which consisted of microscopical striated droplets. The mother liquor on further concentration gave a second and then a

third deposit, all of identical appearance. The combined picrates were washed with ether until the washings became practically colorless. The substance was recrystallized out of 95 per cent alcohol three times. The melting point of the substance was the following: at 140°C. (not corrected) it became transparent, at about 180°C. it began to darken, and at 200°C. it decomposed with the evolution of gas. A sample of cytidine picrate of our collection which was recrystallized in the same manner behaved identically.

For measuring the optical rotation the picrate was dissolved in a warm solution of 1.0 per cent hydrochloric acid, filtered from picric acid which separated on cooling, and the filtrate was used for the measurement. The value found was

$$[\alpha]_D^{20} = \frac{+0.19 \times 100}{1 \times 1} = +19.0.$$

A sample of cytidine picrate previously prepared gave the value

$$[\alpha]_D^{20} = \frac{+0.20 \times 100}{1 \times 1} = +20.0.$$

The analysis of the substance gave the following results.

0.1122 gm. of the substance gave 17.4 cc. of nitrogen at $T° = 21°C.$ and $P = 760$.

0.0902S of the substance gave 0.1392 gm. of CO_2 and 0.0322 gm. of H_2O.

	Calculated for $C_9H_{13}O_5N_3.C_6H_2(NO_2)_3OH$.	Found.
C	38.14	37.94
H	3.40	3.57
N	17.79	17.74

Thus the product of hydrolysis was cytidine where cytosine was expected. No explanation can be given for the unexpected result. Possibly the sulfuric acid solution employed in the experiment contained less than 10 per cent of sulfuric acid. However, for the proof of the nature of the nucleotide the isolation of either cytosine or cytidine has the same value. No uridine was found in the mother liquors from the picrate. In fact on evaporation to dryness and extraction with ether the residue was insignificant.

LIPOIDS OF THE HEART MUSCLE.

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(Received for publication, June 30, 1919.)

INTRODUCTORY REMARKS.

BY P. A. LEVENE.

The work on the phosphatides of the heart muscle has made us see the old distinctions between individual phosphatides in a novel light. Prior to this work most workers distinguished phosphatides first by their solubility, and second by the ratio of the elements of nitrogen and phosphorus.

By their solubility all unsaturated phosphatides were generally classified into three fractions: (1) lecithin, the fraction soluble in ether and alcohol; (2) cephalin, the fraction soluble in ether and insoluble in alcohol, hence, obtained by precipitating the ethereal solution with alcohol; and (3) acetone-soluble fraction.

The lecithin group was regarded as consisting practically entirely of lecithin.

The cephalin fraction of individual organs was claimed to consist of substances varying in their composition with the variation of the organ.

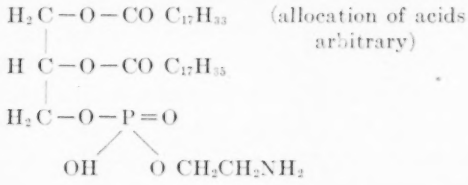
The same applied to the acetone-soluble fraction.

Through the work done in this laboratory evidence was furnished in favor of the view that both the lecithin and acetone-soluble fractions are composed of a mixture in varying proportions of lecithin and cephalin, each respectively having the structure as required by theory. The mixture can be separated into its component parts by reducing the crude material with hydrogen gas in the presence of colloidal palladium, and subsequently separating hydrocephalin from hydrolecithin by fractional precipitation out of organic solvents.

On the other hand, the so called cephalin fraction was proved to consist of a mixture of fragments (of various size) of both lecithin and of cephalin. True, in the cephalin fraction the cephalin fragments predominate over those of lecithin, and *vice versa*, in the lecithin fraction the predominating part is unchanged lecithin. But it is also true that the generally accepted view on the two fractions "lecithin" and "cephalin" has to be radically changed and that part of the ether-soluble material which is also soluble in alcohol should be regarded as the fraction containing the unaltered lecithin and cephalin, and the fraction which is soluble in ether and insoluble in alcohol as consisting principally of mixtures of fragments of lecithin and cephalin. This contention has been proved definitely for the lipoids of the heart muscle. There is reason to believe that the same applies to all other organs. The correctness of this contention is being further tested in this laboratory.

I. Hydrolecithin and Hydrocephalin of Heart Muscle.

In previous communications by Levene and West[1,2] on the phosphatides of the egg yolk, it was shown that the so called lecithin was a mixture of lecithin and cephalin. When reduced by means of hydrogen in the presence of colloidal palladium the material was fractionated into hydrocephalin and hydrolecithin. Through that observation proof was furnished in support of the generally assumed structural formula of cephalin, as follows:



The analysis of the so called lecithin fraction obtained from the heart muscle also consists of a mixture of the same two components. When the lecithin fraction was reduced by means of hydrogen in the presence of colloidal palladium a product was

[1] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1918, xxxv, 285.
[2] Levene, P. A , and West, C. J., *J. Biol. Chem.*, 1918, xxxiv, 175.

obtained which showed a nitrogen distribution $\frac{NH_2N}{\text{Total N}} = \frac{31}{100}$; thus containing nearly one-third of cephalin. The material had all the properties of the crude hydrolecithin obtained from egg yolk. By the process which had been applied for fractionation of the latter material the crude hydrolecithin of the heart muscle was fractionated into two fractions, one consisting principally of hydrocephalin (77 per cent), and the other principally of hydrolecithin (76 per cent). Further purification to substances of greater purity is only a question of material and work.

The composition of the respective samples was as follows:

	Hydrolecithin. $C_{44}H_{90}O_9NP$		Hydrocephalin. $C_{42}H_{82}O_9NP$	
	Calculated.	Found.	Calculated.	Found.
C	65.37	65.86	65.80	65.50
H	11.23	11.39	11.05	10.36
N	1.74	1.86	1.87	1.80
P	3.84	4.06	4.15	4.29
$\frac{NH_2N}{\text{Total N}}$	$\frac{0}{100}$	$\frac{35}{100}$	$\frac{100}{100}$	$\frac{77}{100}$

On repeated recrystallization:

$\frac{NH_2N}{\text{Total N}}$	$\frac{24}{100}$

$$[\alpha]_D^{20} = +4.0 \qquad [\alpha]_D^{20} = +3.3$$

These observations contribute to the evidence in favor of the structural formula of cephalin. The observations are important also from the view-point of another more general consideration. An opinion has been expressed with particular emphasis by Fränkel and Linnert[3] that individual organs of the same animal contain specific phosphatides and further, that identical organs of animals of different species also contained distinct phosphatides. The experience gained in this laboratory, however, has been that as the methods of isolation of the individual lipoids are being perfected and as the knowledge of the structure of individual substance is making progress, so the conviction grows

[3] Fränkel, S., and Linnert, K., *Biochem. Z.*, 1910, xxiv, 270.

that the number of individual lipoids is rather limited, and that practically all animal organs contain the same lipoids. This view has been expressed by one of us on several other occasions.

EXPERIMENTAL.

160.0 gm. of lecithin with a ratio of $\frac{NH_2N}{\text{Total N}} = \frac{32}{100}$ were reduced in the same manner as described by Levene and West. The yield of reduced material after one reduction was 70.0 gm. These were dissolved in hot 95 per cent alcohol and allowed to stand at 10° for 24 hours. 44.0 gm. of the substance settled out on cooling. This material had a ratio of $\frac{NH_2N}{\text{Total N}} = \frac{31}{100}$. This material was recrystallized out of a solvent consisting of equal parts of chloroform and methylethyl ketone. 15 parts of the solvent were used for 1 part of the material. The process was repeated once. The final yield was 25.0 gm. This material was then dissolved in hot chloroform and the solution poured into three volumes of 99.5 per cent alcohol. The precipitate was filtered immediately; the yield was 5.0 gm.

The substance obtained in this manner had the following composition:

0.0992 gm. of substance gave 0.2336 gm. of CO_2, 0.0910 gm. of H_2O, and 0.0115 gm. of ash.

0.127 gm. of substance used for Kjeldahl nitrogen estimation required for neutralization 1.60 cc. of 0.1 N acid.

0.1622 gm. of substance gave on fusion 0.0245 gm. of $Mg_2P_2O_7$.

2 gm. were hydrolyzed and made up to 20 cc. 5 cc. of the solution required 1.05 cc. of 0.1 N acid; 2 cc. of the solution gave 0.81 cc. of N at 26°, P = 760.7 mm.

The rotation of the substance in chloroform was

$$[\alpha]_D^{25} = \frac{+0.10 \times 100}{1 \times 3} = +3.3$$

Since this rotation differed numerically from that made on the hydrocephalin and since the measurements in the present work were made under slightly different conditions than those of the previous year, the measurement of the rotation of the hydrolecithin from the egg yolk was repeated under the present conditions.

The result was as follows:

$$[\alpha]_D^{25} = \frac{+0.11 \times 100}{1 \times 3} = +3.6$$

The mother liquors resulting from crystallization of the material out of solution of chloroform and methylethyl ketone were concentrated and recrystallized out of alcohol. The yield was 10.0 gm. The substance had the following composition:

0.0988 gm. of the substance gave 0.2388 gm. of CO_2, 0.1006 gm. of H_2O, and 0.0101 gm. of ash.

0.1868 gm. of the substance used for Kjeldahl nitrogen estimation required for neutralization 2.45 cc. of 0.1 N acid.

0.1586 gm. of the substance gave on fusion 0.0228 gm. of $Mg_2P_2O_7$.

After repeated recrystallization the ratio of $\frac{NH_2N}{\text{Total N}} = \frac{24}{100}$.

2 gm. of the substance were hydrolyzed and made up to 25 cc.

5 cc. of the solution required 2.15 cc. of 0.1 N acid.

2 cc. of the solution gave 0.56 cc. N at 31°. $P = 756$ mm.

$$\frac{NH_2N}{\text{Total N}} = \frac{24}{100}$$

The rotation of the substance in chloroform was

$$[\alpha]_D^{25} = \frac{0.12 \times 100}{1 \times 3} = +4.0.$$

II. Acetone-Soluble Phosphatides.

It was shown by several observers that the acetone ethereal mother liquors, resulting from precipitation of an ethereal solution of lecithin by acetone, contained phosphatides. Fränkel and his pupils claimed to have discovered among them a variety of new phosphatides. MacLean on the contrary expressed the view that these phosphatides consisted in the main of lecithin.[4] The conclusions of MacLean seemed more acceptable to us particularly in the light of the observation of Levene and West on the phosphatide obtained from egg yolk.[2]

Also in the acetone residue from the heart muscle the phosphatides consisted principally of lecithin and secondarily of a

[4] MacLean, H., *Biochem. J.*, 1914, viii, 453.

small admixture of cephalin. The process of preparation was slightly modified from that described by Levene and West.

The lipoids were prepared in the form of the cadmium chloride salt. The ratio $\frac{NH_2N}{\text{Total N}} = \frac{1}{100}$.

The optical rotation of the cadmium salt was: $[\alpha]_D^{25} = +4.4$.

EXPERIMENTAL.

The acetone-ether solutions were concentrated under reduced pressure to a thick syrup. This was slightly diluted with alcohol and an alcoholic solution of cadmium chloride was added as long as a precipitate formed. The precipitate which had at first a very sticky character was extracted with acetone five times until it settled as a fine dry powder. This substance had the following composition.

0.1757 gm. of the substance employed for Kjeldahl nitrogen estimation required 1.95 cc. of 0.1 N acid for neutralization; N = 1.56 per cent.

0.2548 gm. of the substance gave on fusion 0.0270 gm. of $Mg_2P_2O_7$; P = 2.98 per cent. The ratio of $\frac{NH_2N}{\text{Total N}} = \frac{7}{100}$.

This was suspended in ether and water was added gradually in very small portions until the solution of the salt was completed. The solution was then poured into alcohol. This operation was repeated several times.

The final product had the following composition.

0.100 gm. of the substance gave on combustion 0.1671 gm. of CO_2, 0.0634 gm. of H_2O, and 0.0258 gm. of ash.

0.251 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 2.75 cc. of 0.1 N acid.

0.1700 gm. of the substance gave on fusion 0.0195 gm. of $Mg_2P_2O_7$.

0.500 gm. of the substance gave 0.1506 gm. of $CdSO_4$.

2.0 gm. of the substance were hydrolyzed with hydrochloric acid as usual. The solution was neutralized and made up to 20.0 cc.

5 cc. of this solution required for neutralization 4.25 cc. of 0.1 N acid.

2 cc. of the solution gave 0.06 nitrogen gas at $T° = 29°C.$ and $P = 759$ mm.

	Calculated for $C_6H_{12}O_7N\,P\,Cd_2Cl_2$.	Found.
C	45.13	45.57
H	7.35	7.09
N	1.19	1.53
P	2.65	3.09
Cd	19.20	16.21
NH_2N	0	1
Total N	100	100

Thus the substance consisted practically of the dicadmium salt, containing perhaps an insignificant proportion of the monocadmium derivative.

The optical rotation of the substance was

$$[\alpha]_D^{25} = \frac{+0.11 \times 100}{0.5 \times 5} = +4.40.$$

CEPHALIN.

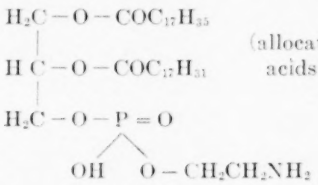
VI. THE BEARING OF CUORIN ON THE STRUCTURE OF CEPHALIN.

By P. A. LEVENE and S. KOMATSU.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 30, 1919.)

The hitherto isolated components of cephalin are phosphoric acid, glycerol, aminoethanol, and stearic and linoleic acids. A molecule composed of equimolecular proportions of these substances may be represented by the following graphic formula.



(allocation of fatty acids arbitrary)

This molecule requires the following percentage composition of the elements entering into it.

	C	H	N	P
Per cent	66.16	10.57	1.88	4.18

The values generally found on analysis by various investigations were

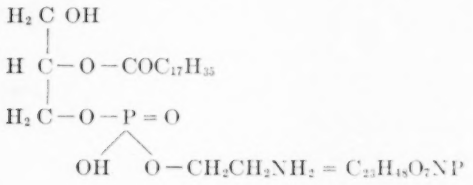
	C	H	N	P
Per cent	60.13	9.50	1.80	3.70

This discrepancy between theory and fact never received a satisfactory explanation.

A priori three alternative explanations were possible: first, cephalin possessed a structure different from the one expressed by the given formula; second, the cephalin isolated by the usual methods represented a substance modified in the process of preparation; and third, the substance was a mixture of true cephalin with some other substance of unknown composition.

Recently Levene and West[1] have prepared a reduced cephalin which possessed an elementary composition required by the structural formula given above. This find excludes the first of the three alternatives, and the choice remains between the second and the third.

The present communication offers an explanation to the puzzling analytical data obtained on cephalin by all workers beginning with Thudichum. In the light of the work to be reported here both the second and the third alternatives combine to bring about the known empirical composition of the so called cephalin. It appears that cephalin is a mixture of true cephalin with decomposition products of it. One of the decomposition products is cephalin from which one fatty acid has been removed either by the chemical manipulations or by enzymes. A substance of this nature should have the following composition.



This substance should give the following analytical values.

	C	H	N	P
Per cent	57.3	9.9	2.7	6.4

A substance in its elementary composition approaching this hypothetical fragment of cephalin was isolated. It contained

	C	H	N	P
Per cent	56.93	9.23	2.14	6.05

Substances were obtained also which consisted apparently of products of deeper deterioration of the original cephalin molecule, thus a fraction was isolated which analyzed as follows

	C	H	N	P
Per cent	54.43	8.70	1.46	6.42

which was perhaps a mixture of the monostearyl-aminoethanol derivative of glycerophosphoric acid with stearyl-glycerophos-

[1] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1918, xxxv, 285.

phoric acid and perhaps with some glycerophosphoric acid. The theory for monostearyl-glycerophosphoric acid requires the following values.

Calculated for $C_{21}H_{45}O_5NP$ (molecular weight 452)

	C	H	N	P
Per cent	55.7	9.57	0	6.85

In other words the so called cephalin consists of true cephalin and of all the products of its intermediate hydrolysis, namely, of monostearyl-glycerophosphoric aminoethanol ester, monostearyl-glycerophosphoric acid, and glycerophosphoric acid. This conclusion was arrived at in course of an investigation into the chemical nature of cuorin.

Cuorin.

Cuorin was described by Erlandsen[2] as an individual phosphatide differing from both lecithin and cephalin. By the method of preparation and by its physical properties cuorin showed close resemblance to cephalin. The difference lay in the values of the elements nitrogen and phosphorus present in the molecules of the two substances. In cephalin the values are N = 1.88, P = 4.18 and the ratio N: P = 1: 1, whereas in cuorin the values are 1 and 4 respectively and the ratio N: P = 1: 2.

However there were reasons to suspect that cuorin was not an individual substance but a mixture of cephalin with other substances. MacLean,[3] who described substances resembling Erlandsen's cuorin, expressed, in his monograph on lecithin, doubts as to the existence of cuorin as an individual substance. These doubts were accentuated by the observations made in the course of the present work. First, by apparently identical methods of preparation substances were obtained with the composition approaching either that of cuorin or that of the so called cephalin. Second, from a substance with the composition of cuorin, depending on the mode of treatment could be obtained a large proportion of a substance resembling cephalin or *vice versa*. Third, as in crude cephalin or lecithin the basic component of the substance

[2] Erlandsen, A., *Z. physiol. Chem.*, 1907, li, 71.

[3] Maclean, H., Lecithin and allied substances. The lipins, *Monographs on Biochemistry*, London, 1918, 52.

was not uniform, but consisted of a mixture of choline and aminoethanol and on fractionation the individual fraction did not contain the bases in stoichiometric proportions. On the contrary, the fractions more soluble in alcohol contained progressively smaller proportions of aminoethanol with the increase of their solubility, and *vice versa*. Fourth, the proportion of fatty acids varied irregularly in various fractions.

The greatest part of cuorin, however, is made up of the material generally called crude cephalin. Thus every sample of cuorin, if purified by precipitation out of aqueous solution by hydrochloric acid, contains the elements nitrogen and phosphorus in ratio of N: P = 1: 1. A sample of material was prepared according to the process of Erlandsen, and it had the composition:

	C	H	N	P
Per cent	61.85	8.42	1.66	4.60

and the ratio N: P = 1: 1.25. From this substance at will could be obtained either a substance analytically resembling cuorin or cephalin.

Thus when allowed to settle out on cooling out of hot methylethyl ketone, and subsequently allowed to settle out on cooling out of hot ethylacetate a material was obtained having the values of the elements of nitrogen and phosphorus 1.22 per cent and 4.15 per cent respectively, or the ratio N: P = 1: 1.54. When the same material was purified by the hydrochloric acid process a substance was obtained which was separated into two fractions, one having N = 2.04 and P = 4.07; the ratio N: P = 1: 1 approximately, and the other N = 1.61 and P = 4.13; the ratio N: P = 1: 1.17. In another experiment the material having N = 1.6 and P = 4.4 and N: P = 1: 1.25 was separated by means of methylethyl acetone into two fractions, one having N = 1.6 and P = 4.0, N: P = 1: 1.13; and the other having N = 1.86 per cent and P = 8.40 per cent, N: P = 1: 2.03.

The basic components of the individual samples varied considerably. Thus, the proportion of $\frac{NH_2N}{\text{Total N}}$ varied from 0.70 to 0.45. Therefore, it seemed clear that cuorin was a mixture. The question then arose as to the nature of the materials which make up the so called cuorin.

It was concluded that these were the same fragments of cephalin and lecithin which make up the so called crude cephalin. Some of the fragments were enumerated in the opening chapter of this communication.

Reduction of the Cephalin Fraction of Cuorin.

For convenience of the work on the nature of the material obtained from so called cuorin and resembling cephalin the material was reduced by means of hydrogen in the presence of colloidal palladium. It was found that the reduction proceeded more satisfactorily when the substance had been purified by the hydrochloric acid process. Just as in the earlier experience of Levene and West[4] it was also found that the reduction of cephalin proceeded at a very slow rate and that the volume of absorbed hydrogen was comparatively small.

The main product obtained after reduction had an elementary composition nearly identical with that obtained by different investigators on unreduced cephalin. The values obtained by Thudichum[5] and by Levene and West on unreduced cephalin were

	C	H	N	P
Thudichum	60.00	9.38	1.68	4.27
Levene and West	60.33	9.62	1.78	3.60

and on reduced cephalin

Levene and Komatsu	59.47	9.41	1.68	4.17

The material contained about 92 per cent of its nitrogen in the form of amino nitrogen. Thus it seemed as if some factor was interfering with the reduction of cephalin. Two alternative assumptions were capable of explaining this observation; first, that the unsaturated fatty acid through oxidation was converted into hydroxyacid which could not naturally be reduced by hydrogen in the presence of a catalyst; second, that the unsaturated fatty acid was prevented from absorbing hydrogen by the presence of an impurity acting as a catalytic poison.

[4] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1916, xxiv, 45.

[5] Thudichum, J. L. W., The chemical constitution of the brain, London, 1884, 57.

Under the first assumption an hydroxyacid should be found among the hydrolytic products of the above material, under the second, unsaturated fatty acids. Neither one of the two expectations was realized. On hydrolysis of the material there was found only one fatty acid, namely, stearic acid. Furthermore, the value of the fatty acids in the material was found to be 64.16 per cent. A value of 63.40 per cent was previously found by Levene and West on unreduced cephalin. The theory for the cephalin molecule containing two molecules of fatty acids requires 75.90 per cent of the acids, and the theory of the fragment with one fatty acid requires 58.84 per cent. On the basis of these considerations it was assumed that the material was a mixture of cephalin with its fragments. Indeed on modifying the mode of treatment there were obtained fractions of varying composition.

	C	H	N	P
I	62.51	9.68	1.58	4.01
II	62.13	9.36	2.04	4.17
III	61.94	9.47	2.43	5.81
IV	61.03	10.11	2.00	5.15
V	56.93	9.23	2.14	6.05
VI	54.43	8.70	1.46	6.42

Of these, (V) in composition approaches monostearyl-glycerophosphoriaminoethanol ester. Taking into consideration that every one of these fragments is composed of some or of all of the following radicles, that of phosphoric acid, of glycerol, of stearic acid, and of aminoethanol; taking further into consideration that, judging from the elementary analysis, the radicles are present in proportions that have no stoichiometric relationship, one is inclined to assume that every one of the fractions is a mixture of the fragments of cephalin, also that the so called cephalin (C = 60.0 per cent approximately) is a mixture of such fragments, and that cuorin differs from cephalin only inasmuch as it contains a larger proportion of lower fragments.

EXPERIMENTAL.

Preparation of Crude Cuorin.

Heart muscle was minced in meat chopping machines, dried in vacuum driers, and pulverized. The dry powder was extracted with ether containing 7.0 per cent water, since it was found that

anhydrous ether extracted only minimal quantities of phosphatides. The ethereal solution was concentrated to small volume and to it dry acetone was added as long as a precipitate was formed. The precipitate was dried under diminished pressure over sulfuric acid, redissolved in ether, and allowed to stand at 0°C. over night to allow the "white matter" to settle out. The ethereal solution was again concentrated and treated with acetone. The operation was repeated as many times as the new ethereal solution on standing at low temperature still formed a sediment of white matter. The final product obtained in this manner was further treated according to Erlandsen, and the product insoluble in 95 per cent alcohol at 60°C. was expected to be cuorin. However, the ratio of the elements nitrogen to phosphorus was found greater than the one required for cuorin and smaller than that for cephalin, namely, $\frac{N}{P}$ was greater than $\frac{1}{2}$ and smaller than 1. Under similar treatment Erlandsen obtained a cuorin having the elements nitrogen and phosphorus in the ratio $\frac{N}{P} = \frac{1}{2}$.

Analysis of Crude Cuorin.

Sample A.—0.2519 mg. of the substance used for Kjeldahl nitrogen estimation required 2.9 cc. of 0.1 N acid for neutralization, N = 1.6 per cent.

0.2493 gm. of the substance on fusion gave 0.0395 gm. of $Mg_2P_2O_7$, P = 4.4. Ratio of N : P = 1 : 1.25.

2 gm. were used for the estimation of the distribution of nitrogen. The filtrate after hydrolysis was made up to 15 cc. 5 cc. used for total nitrogen estimation required for neutralization 1.2 cc. of 0.1 N acid. 2 cc. used for amino nitrogen estimation gave 0.85 cc. of nitrogen at $T = 22°$, $P = 758.6$ mm. $\frac{NH_2N}{\text{Total N}} = \frac{70}{100}$.

Sample B.—The analysis of this sample gave the following results. 0.100 gm. of the dry material gave 0.2180 gm. of CO_2, 0.0724 gm. of H_2O, and 0.014 gm. of ash.

0.2885 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 3.3 cc. of 0.1 N acid.

0.3012 gm. of the substance gave on fusion 0.0477 gm. of $Mg_2P_2O_7$.

$$\frac{NH_2N}{\text{Total N}} = \frac{65}{100}$$

	C	H	N	P
Hence ash-free per cent.....	61.85	8.42	1.66	4.60

Fractionation and Purification of Crude Cuorin.

The first step in fractionation of the crude cuorin was accomplished by means of methylethyl ketone. The material dried under diminished pressure was dissolved in the boiling reagent. Under these conditions a small part remained insoluble. This fraction obtained from Sample A had a composition of N = 1.86 per cent and P = 8.40 per cent, from Sample B, N = 2.54 per cent and P = 5.74 per cent. The soluble part on cooling formed a precipitate. In Sample A this fraction had the following composition.

0.3452 gm. of the substance employed from Kjeldahl nitrogen estimation required for neutralization 3.9 cc. of 0.1 N acid.

0.2924 gm. of the substance gave on fusion 0.042 gm. of $Mg_2P_2O_7$.

$$N = 1.6 \text{ and } P = 4.0 \text{ per cent; } \frac{N}{P} = \frac{1}{1.13}; \frac{NH_2N}{\text{Total N}} = \frac{70}{100}$$

The second step in the purification of the fraction soluble in hot methylethyl ketone was accomplished in the following two manners: (*a*) it was recrystallized out of dry ethylacetate; (*b*) it was taken up in water and placed in a shaking machine until the mixture acquired the character of an opalescent viscous liquid. From this the substance was precipitated by means of 10 per cent hydrochloric acid. The precipitate was separated either by filtration or by centrifugalization. The sediment was washed with acetone, then dissolved in ether, and poured into alcohol. The material obtained by the first process contained the elements nitrogen and phosphorus in the following proportions.

0.3055 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 2.58 cc. of 0.1 N acid.

0.2479 gm. of the substance gave on fusion 0.0382 gm. of $Mg_2P_2O_7$.

$$N = 1.18 \text{ per cent and } P = 4.31 \text{ per cent, } \frac{N}{P} = \frac{1}{1.66}$$

This material was dissolved in hot ethlyacetate and allowed to cool. A precipitate formed which was again dissolved in ether and precipitated by means of acetone. The substance had the following composition.

0.1008 gm. of the dry material gave 0.2170 gm. of CO_2, 0.0824 gm. of H_2O, and 0.013 gm. of ash.

0.2938 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 2.5 cc. of 0.1 N acid.

0.2888 gm. of the substance gave on fusion 0.0413 gm. of $Mg_2P_2O_7$.

	C	H	N	P	$\frac{N}{P}$
Hence ash-free per cent..	61.33	10.08	1.22	4.15	$\frac{1}{1.54}$

The material obtained by the hydrochloric acid process gave two fractions, one formed on the precipitation of the ethereal solution by 95 per cent alcohol (A_1) and the other by concentrating the mother liquor and precipitating it with acetone (B_1). Fraction (A_1) was dissolved in ether and centrifugalized to remove the very small insoluble part. The clear filtrate was concentrated and precipitated with acetone. It had the following composition.

0.2943 gm. of the substance gave on fusion 0.0424 gm. of $Mg_2P_2O_7$.

0.2530 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 3.7 cc. of 0.1 N acid.

$$\frac{NH_2N}{\text{Total N}} = \frac{70}{100}. \quad N = 2.04 \text{ per cent and } P = 4.07 \text{ per cent.}$$

Fraction (B_1) had the following composition.

0.2520 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 2.9 cc. of 0.1 N acid.

0.2503 gm. of the substance gave on fusion 0.0370 gm. of $Mg_2P_2O_7$.

$$\frac{NH_2N}{\text{Total N}} = \frac{44}{100}. \quad N = 1.61 \text{ per cent and } P = 4.13 \text{ per cent.}$$

Reduction of Fraction A.

It was found by experiment that the so called cuorin absorbed hydrogen with greater readiness, if prior to reduction it was purified by the hydrochloric acid process. For this reason all material used for reduction was purified in this manner. The material was dissolved in ether in 10 gm. lots, to the solution a little glacial acetic acid was added, and the catalyst was taken up in a few cc. of water and introduced into the apparatus. The reaction proceeded about 50 hours. In the course of the reduction some solid material settled out. The reaction product was placed at 0°C. over night and filtered. The residue was dissolved in boiling 95 per cent alcohol and allowed to cool. A slightly colored

precipitate is thus formed. This is dissolved in chloroform (using 1 cc. of chloroform for 0.5 gm. of the substance) and the solution poured into 5 volumes of 99.5 per cent alcohol. The precipitate thus formed is redissolved and reprecipitated twice. The final substance had the following composition.

0.102 gm. of substance gave 0.2088 gm. of CO_2, 0.0796 gm. of H_2O, and 0.0152 gm. of ash.

0.1468 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 1.6 cc. of 0.1 N acid.

0.1493 gm. of the substance on fusion gave 0.0204 gm. of $Mg_2P_2O_7$.

	C	H	N	P	$\frac{NH_2N}{Total\ N}$
Found per cent.....	59.47	9.41	1.68	4.17	$\frac{92}{100}$

Fatty Acids in This Material.

4.8 gm. of the substance were taken up in 80.0 cc. of 3 per cent sulfuric acid and heated in a sealed tube with shaking for 24 hours at 105°C. At the end of the reaction the fatty acid was extracted with ether and the ethereal solution evaporated to dryness. The residue was extracted with acetone. The filtrate was again concentrated to a solid cake. Dried to constant weight it contained 3.08 gm. Hence the proportion of fatty acids in the material was $\frac{3.08}{4.8} = \frac{64.16}{100}$.

For identification of the fatty acids 15.0 gm. of the material were hydrolyzed in the same manner as above, and the fatty acids converted into the barium salts under the conditions given by Levene and Meyer.[6] The barium salts were extracted with ether, in which the barium salts of unsaturated fatty acids are soluble. However, the ethereal extract on evaporation left practically no residue. The barium salts were then converted into the free acids, these converted into the lead salts which were again converted into the free acids, and the free acids recrystallized out of acetone. The acid had the melting point, M. P. = 68–69° (corrected) and had the following composition.

0.0992 gm. of substance gave on combustion 0.2765 gm. of CO_2 and 0.1106 gm. of H_2O.

[6] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1917, xxxi, 631.

	Calculated for $C_{18}H_{36}O_2$.	Found.
C	76.06	76.00
H	12.68	12.48

The molecular weight of the substance was the following.

0.6032 gm. of substance required for neutralization 21.1 cc. of 0.1 N acid; M. P. = 285.

Glycerol Estimation.

As in the previous work the Zeisel-Fanto[7] method was employed for the estimation of glycerol. The temperature of the oil bath was maintained at 125–128°C.

0.3153 gm. of the material gave 0.0940 gm. of AgT which corresponds to 11.7 per cent of glycerol. The theory for cephalin requires 12.32 per cent and for the monoacyl derivative 19.12 per cent. Levene and West found for cephalin 10 per cent, and Foster[8] 8.21 per cent. The method can scarcely be regarded as quantitative, but the value for glycerol obtained by us on material with a lower content of carbon is higher than the values obtained on cephalin with a higher carbon content. This result is to be expected in the light of the assumption made by us for the constitution of the so called cephalin.

Fractionation of the Reduced Material.

In a second experiment the reduced material, which had settled on cooling of a hot solution in 95 per cent alcohol, was fractionated by means of 99.5 per cent alcohol.

One part (*a*) remained insoluble in hot 99.5 per cent alcohol and the other (*b*) remained in solution in that solvent but settled out on cooling.

Each one of the two fractions was then purified by dissolving in chloroform (1 gm. in 2 cc.) and pouring into three volumes of 99.5 per cent alcohol Fraction (*a*) had the following composition.

0.1028 gm. of the substance gave 0.1962 gm. of CO_2, 0.0760 gm. of H_2O, and 0.0190 gm. of ash.

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[7] Zeisel, S., and Fanto, R., *Z. anal. Chem.*, 1903, xlii, 549.

[8] Foster, M. L., *J. Biol. Chem.*, 1915, xx, 403.

0.1445 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 1.44 cc. of 0.1 N acid.

0.1498 gm. of the substance gave on fusion 0.0329 gm. of $Mg_2P_2O_7$.

	C	H	N	P
Hence ash-free per cent.....	54.43	8.70	1.46	6.42

Fraction (*b*) had the following composition.

0.1006 gm. of the substance gave 0.2222 gm. of CO_2, 0.0816 gm. of H_2O, and 0.0124 gm. of ash.

0.1594 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 2.25 cc. of 0.1 N acid.

0.1562 gm. of the substance gave on fusion 0.025 gm. of $Mg_2P_2O_7$.

	C	H	N	P	$\frac{NH_2N}{Total\ N}$
Hence ash-free per cent...	62.13	9.36	2.04	4.17	$\frac{90}{100}$

In a third experiment the fractionation was carried out by means of butyl alcohol. The material used on this occasion was the fraction soluble in hot 95 per cent alcohol and insoluble in the cold.

The substance was dissolved in boiling butyl alcohol and allowed to cool at room temperature (about 25–28°C.) when a sediment (a_1) formed. The mother liquor on standing at about 10°C. gave rise to a second precipitate (b_1). (a_1) had the following composition.

0.1028 gm. of the substance gave 0.2278 gm. of CO_2, 0.0910 gm. of H_2O, and 0.0130 gm. of ash.

0.1506 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 2.1 cc. of 0.1 N acid.

0.1504 gm. of the substance gave on fusion 0.0274 gm. of $Mg_2P_2O_7$.

	C	H	N	P	$\frac{NH_2N}{Total\ N}$
Hence ash-free per cent...	61.03	10.11	2.00	5.15	$\frac{60}{100}$

(b_1) contained the elements of nitrogen and phosphorus in the following proportions.

0.1690 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 2.55 cc. of 0.1 N acid.

0.1647 gm. of the substance gave on fusion 0.0196 gm. of $Mg_2P_2O_7$.

	N	P	$\frac{NH_2N}{Total\ N}$
Hence, found per cent.....	2.11	3.33	$\frac{30}{100}$

(a_1) on renewed purification through butyl alcohol again gave two fractions, the less soluble (a_2) and the more soluble (b_2). (a_2) had the following composition.

0.1022 gm. of the substance gave 0.206 gm. of CO_2, 0.0838 gm. of H_2O, and 0.016 gm. of ash.

0.1430 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 2.25 cc. of 0.1 N acid.

0.1367 gm. of the substance gave on fusion 0.0266 gm. of $Mg_2P_2O_7$.

	C	H	N	P	$\frac{NH_2N}{Total\ N}$
Hence ash-free per cent....	61.94	9.47	2.43	5.81	$\frac{85}{100}$

(b_2) gave the following analytical values.

0.1008 gm. of the substance gave 0.2172 gm. of CO_2, 0.1172 gm. of H_2O, and 0.0126 gm. of ash.

0.1474 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 2.2 cc. of 0.1 N acid.

0.1512 gm. of the substance gave on fusion 0.029 gm. of $Mg_2P_2O_7$.

	C	H	N	P	$\frac{NH_2N}{Total\ N}$
Hence ash-free per cent...	59.19	12.92	2.09	5.41	$\frac{78}{100}$

All the crude reduced material when it is taken up in hot 95 per cent alcohol leaves a small insoluble residue which adheres to the palladium tenaciously. It was found possible to separate it from the palladium by means of a hot solution of the following solvent: 1 part by volume of chloroform, 1 part of ether, and 1 part of 95 per cent alcohol. On standing at room temperature it formed a precipitate (a_3) and filtrate (b_3). (a_3) was dissolved in chloroform and precipitated by means of three volumes of 99.5 per cent alcohol. The substance obtained in this manner had the following composition.

0.0998 gm. of the substance gave 0.2274 gm. of CO_2, 0.0810 gm. of H_2O, and 0.0134 gm. of ash.

0.1350 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 1.6 cc. of 0.1 N acid.

0.1540 gm. of the substance gave on fusion 0.0211 gm. of $Mg_2P_2O_7$.

	C	H	N	P	$\frac{NH_2N}{Total\ N}$
Hence ash-free per cent....	62.51	9.68	1.58	4.01	$\frac{95}{100}$

d-CHONDROSAMINO- AND d-CHITOSAMINOHEPTONIC ACIDS.

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The theoretical interest attached to the preparation of these substances was discussed in previous communications.[1] The behavior of them to nitrous acid, it is hoped, may furnish valuable information in connection with the problem of the allocation of the amino group in the α-aminohexoses and α-aminohexosaminic acids.

As regards the preparation of the heptonic acids, the present communication contains specific directions leading to satisfactory yields of chondrosaminoheptonic acid, and simplified directions, as compared with the original, for the preparation of chitosaminoheptonic acids. Since the product obtained on the action of prussic acid on hexosamines is a mixture of two epimers, it became necessary to separate the mixture into its components. This part of the problem was solved satisfactorily.

The d-chondrosaminoheptonic acids with the original specific rotation of $[\alpha]_D^{25} = -3.5$ were fractionated into a levo-d-chondrosaminoheptonic acid with the initial rotation $[\alpha]_D^{20} = -8.25$ and with the equilibrium value of $[\alpha]_D^{25} = -13.00$ and a dextro-d-chondrosaminoheptonic acid with a rotation of $[\alpha]_D^{25} = +42.5$ and equilibrium rotation of $[\alpha]_D^{25} = +65.0$. In the original product the levo form predominates.

The d-chitosaminoheptonic acids with the rotation $[\alpha]_D^{20} = +4.0$ and equilibrium value of $[\alpha]_D^{20} = -1.0$ were separated into a dextro-d-chitosaminic acid with initial rotation of $[\alpha]_D^{25} = +6.5$ and equilibrium rotation of $[\alpha]_D^{25} = +2.75$ and a levo-d-chitosaminic acid with initial rotation of $[\alpha]_D^{25} = -7.5$ and equilibrium rotation

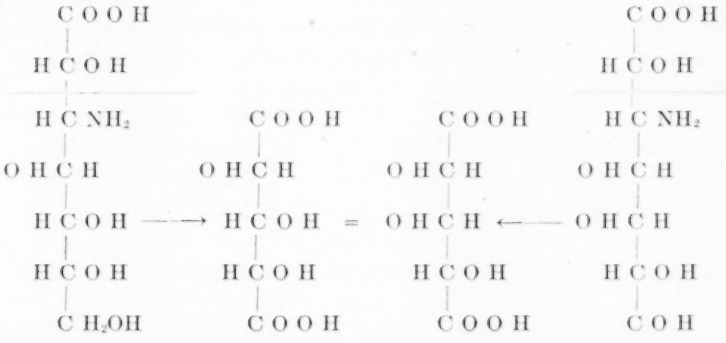
[1] Levene, P. A., *J. Biol. Chem.*, 1916, xxiv, 55; xxvi, 152.

of $[\alpha]_D^{25} = -12.0$. The dextro form predominates in the original product.

The problem of the action of nitrous acid is as yet not brought to a conclusion and is reported here only because one of the authors is compelled to discontinue his cooperation.

It was attempted first to isolate the deaminoheptonic acids, but thus far they were not obtained in a satisfactory physical condition.

On action with nitric acid subsequent to deamination insoluble calcium salts were obtained which gave analytical data satisfactory for the calcium salts of trioxyglutaric acids. Thus: Repeated recrystallization, conversion into lead salt with reconversion into calcium salt, fractionation with lead acetate, and subsequent conversion into calcium salt all led to calcium salts with the values of the elements as required for trioxyglutaric acids. Yet such a conclusion is not acceptable for the reason that both heptonic acids should yield one and the same trioxyglutaric acid, as seen from the following.



Whereas the calcium salts obtained from the two acids possessed a different optical rotation, the rotation of the acid obtained from chondrosaminoheptone was $[\alpha]_D^{25} = +5.0$ initial and $[\alpha]_D^{25} = +1.5$ equilibrium. That of the chitosaminoheptonic $[\alpha]_D^{25} = +10.0$ initial and $[\alpha]_D^{25} = +17.0$ equilibrium. Hence, conditions will have to be discovered for the purification of the dicarboxylic acids arising from deaminoheptonic acids.

EXPERIMENTAL.

Preparation of Chondrosaminoheptonic Acid.

35 gm. of chondrosamine hydrochloride were taken up in 70 cc. of water; 35 cc. of 80 per cent aqueous solution of hydrocyanic acid and 22 cc. of strong ammonia water were added. This mixture was warmed between 42–45° for about 15 minutes. The solution turned dark in color and became viscous. It was cooled in an ice-alcohol bath until the temperature of solution came down to 0°C., when it was transferred into an aqueous solution of 200 gm. of barium hydroxide. The solution was allowed to stand about 1 hour, then boiled on free flame about 3 hours, and distilled under diminished pressure to dryness. The residue was dissolved in 100 cc. of water and the solution was again distilled to dryness; this operation was repeared ten times. By this procedure all ammonia was driven off.

Barium was removed by means of a small excess of sulfuric acid, and the excess of this was removed by means of lead carbonate. The hydrochloric acid was removed by means of $AgCO_3$. The excess of lead and silver was removed by means of hydrogen sulfide gas. The filtrate from silver sulfide was concentrated under diminished pressure to a syrup, and seeded with chondrosaminoheptonic acid. Hot methyl alcohol was added very cautiously until slight opalescence and the solution was allowed to stand about 72 hours at room temperature. (Sometimes the substance crystallized out of aqueous solution.)

The crystalline deposit was filtered, washed with 50 per cent alcohol, 95 per cent alcohol and absolute alcohol successively, and finally with ether. The best yield was 11 gm. In this manner 443 gm. of the substance were prepared.

Levo-d-Chondrosaminoheptonic Acid.

440 gm. of chondrosaminoheptonic acid, which had the following rotation in 2.5 per cent HCl solution

$$[\alpha]_D^{25} = \frac{\text{Initial.}\quad -0.14 \times 100}{1 \times 4} = -3.5$$

were dissolved in 800 cc. of hot water. On standing over night crystals separated out, which had the following rotation.

Initial. Equilibrium (24 hours).

$$[\alpha]_D^{25} = \frac{-0.30 \times 100}{1 \times 4} = -7.5 \qquad [\alpha]_D^{25} = \frac{-0.48 \times 100}{1 \times 4} = -12.00$$

Yield was 344 gm.

The 344 gm. of heptonic acid were dissolved in 800 cc. of hot water and 391 gm. of crystals were obtained. The rotation in 2.5 per cent HCl solution was the following.

Initial. Final (24 hours).

$$[\alpha]_D^{25} = \frac{-0.33 \times 100}{1 \times 4} = -8.25 \qquad [\alpha]_D^{25} = \frac{-0.49 \times 100}{1 \times 4} = -12.25$$

These crystals were dissolved again in 800 cc. of hot water and 265 gm. of crystals were obtained which had the same rotation in 2.5 per cent HCl solution.

Initial. Final (24 hours).

$$[\alpha]_D^{25} = \frac{-0.33 \times 100}{1 \times 4} = -8.25 \qquad [\alpha]_D^{25} = \frac{-0.52 \times 100}{1 \times 4} = -13.00$$

In the mother liquor a further deposit of the same substance settled out. The total yield of levo-chondrosaminoheptonic acid was 313 gm. It crystallized out of water in elongated prisms. Melting point was M. P. = 139°C. (corrected) with decomposition.

Dextro-d-Chondrosaminoheptonic Acid.

The mother liquor of the first separation was concentrated under diminished pressure and a precipitate formed and was filtered off. To the filtrate an equal volume of 95 per cent alcohol was added and the solution was allowed to stand 24 hours. A second precipitate formed and was filtered off again and the solution was poured into absolute alcohol under constant stirring. A gummy precipitate formed which was removed and the solution was concentrated under diminished pressure at room temperature to a syrup. Alcohol was then added and the solution allowed to stand for several days.

Crystals separated which had the following rotation in 2.5 per cent HCl solution

Initial. Final (24 hours).

No. 351

$$[\alpha]_D^{25} = \frac{+0.64 \times 100}{1 \times 4} = +16.0 \qquad [\alpha]_D^{25} = \frac{+1.02 \times 100}{1 \times 4} = +25.50$$

Yield was 1.9 gm.

In the mother liquor a second deposit of 2.3 gm. of crystals formed which had the following rotation in 2.5 per cent HCl solution.

	Initial.	Final (24 hours).
No. 311	$[\alpha]_D^{25} = \frac{+0.50 \times 100}{1 \times 2} = +25.0$	$[\alpha]_D^{25} = \frac{+0.85 \times 100}{1 \times 2} = +42.5$

In the mother liquor a further deposit of crystals formed which had the following rotation in 2.5 per cent HCl solution.

	Initial.	Final (24 hours).
No. 352	$[\alpha]_D^{25} = \frac{+0.85 \times 100}{1 \times 2} = +42.5$	$[\alpha]_D^{25} = \frac{+1.43 \times 100}{1 \times 2} = +71.50$

From the mother liquor of this substance there was obtained on further crystallization a substance with the following specific rotation in 2.5 per cent HCl solution.

	Initial.	Final (24 hours).
No. 407	$[\alpha]_D^{25} = \frac{+0.85 \times 100}{1 \times 2} = +42.5$	$[\alpha]_D^{25} = \frac{+1.30 \times 100}{1 \times 2} = +65.0$

Preparation of Chitosaminoheptonic Acids.

The conditions for the preparations of these acids are more uncertain than of any other substance of this group. At one time, namely in 1915, good yields of the crystalline substance were obtained in the following way. 50.0 gm. of chitosamine hydrochloride were taken up in 100 cc. of water, 18 cc. of an 80 per cent aqueous prussic acid and 25 cc. of ammonium hydroxide were added, and the solution allowed to stand from 24 to 48 hours. The product was then transferred into water containing about 150.0 gm. of barium hydroxide and the solution was boiled over free flame as long as ammonia was still evolved (about 48 hours). The barium and hydrochloric acids were removed in the usual way and the final solution was concentrated under diminished pressure to a thick syrup. This was taken up in a little water, hot 95 per cent alcohol was added as long as a gummy precipitate formed, and the supernatant liquid was decanted and allowed to stand over night at 0°C. A crystalline deposit formed, and

from the mother liquor on concentration and repeated treatment with alcohol, etc., a second crop of crystals was generally obtained. The total yield was about 30 per cent of the employed chitosamine. This material was slightly levorotary.

In 1916 the laboratories were transferred into a new building and all attempts to prepare the material by apparently the same process failed. After many experiments it was finally possible again to obtain the substance under the following conditions. 35 gm. of chitosamine hydrochloride were dissolved in 100 cc. of water; 35 gm. of aqueous 80 per cent prussic acid and 20 cc. of ammonia were added. The mixture was warmed to 30°C. and then allowed to stand at room temperature for 1 hour. The temperature generally remained at that temperature and all the sugar was dissolved in the course of that time. The solution was then transferred into an aqueous solution of barium hydroxide and the further treatment proceeded as above. The final solution was concentrated to a small volume and hot methyl alcohol was added to slight opalescence. The solution was allowed to stand at room temperature over night. A crystalline deposit formed, the yield being about 15 per cent of the employed chitosamine hydrochloride. The specific rotation of this substance was $[\alpha]_D^{20} = +4.0$.

Dextro-d-Chitosaminoheptonic Acid.

130 gm. of chitosaminoheptonic acid were dissolved in four parts of hot water. The original material had the following rotation in 2.5 per cent HCl solution.

	Initial.	Final.
No. 225	$[\alpha]_D^{20} = \frac{+0.16 \times 100}{1 \times 4} = +4.0$	$[\alpha]_D^{20} = \frac{-0.04 \times 100}{1 \times 4} = -1.0$

After standing over night no crystals separated, hence the solution was concentrated under diminished pressure until a crystalline deposit began to form and then allowed to stand over night. 74 gm. of crystals were obtained which had the following rotation in 2.5 per cent HCl solution.

	Initial.
No. 236	$[\alpha]_D^{25} = \frac{+0.23 \times 100}{1 \times 4} = +5.75$

These 74 gm. of heptonic acid were dissolved in 150 cc. of hot water; 43 gm. of crystals settled out which had the following rotation in 2.5 per cent HCl solution.

Initial.	Final (24 hours).
No. 246	
$[\alpha]_D^{25} = \frac{+0.26 \times 100}{1 \times 4} = +6.5$	$[\alpha]_D^{25} = \frac{+0.11 \times 100}{1 \times 4} = +2.75$

These crystals were dissolved again in 86 cc. of hot water and there were obtained 35 gm. of crystals which had the following rotation in 2.5 per cent HCl solution.

Initial.

No. 247

$$[\alpha]_D^{25} = \frac{+0.26 \times 100}{1 \times 4} = +6.5$$

These 35 gm. were again dissolved in 70 cc. of hot water, and there were obtained 28 gm. of crystals which had the following rotation in 2.5 per cent HCl solution.

Initial.

$$[\alpha]_D^{25} = \frac{+0.26 \times 100}{1 \times 4} = +6.5$$

From the mother liquor 37 gm. of the same substance were obtained; the total yield was 65 gm. The substance crystallized out of water in heavy prisms. Melting point was M. P. = 192°C. with decomposition.

Levo-d-Chitosaminoheptonic Acid.

To the mother liquor of the first precipitate hot methyl alcohol was added to a slight opalescence and the solution was allowed to stand 4 hours. A precipitate then separated and was filtered off; the mother liquor was concentrated under diminished pressure and hot alcohol was added. A precipitate was again formed and filtered off and to the mother liquor more alcohol was added. 5 gm. of crystals separated which had the following rotation in 2.5 per cent HCl solution.

Initial.

No. 307

$$[\alpha]_D^{25} = \frac{-0.16 \times 100}{1 \times 4} = -4.0$$

These 5 gm. were dissolved in 5 cc. of hot water. After long standing there were obtained 1.85 gm. of crystals which had the following rotation in 2.5 per cent HCl solution.

Initial.

No. 309

$$[\alpha]_D^{25} = \frac{-0.13 \times 100}{1 \times 2} = -6.5$$

The mother liquor of No. 307 was allowed to stand. On long standing there crystallized 4.75 gm. of a substance with the following rotation in 2.5 per cent HCl solution.

Initial.

No. 261

$$[\alpha]_D^{25} = \frac{-0.11 \times 100}{1 \times 2} = -5.5$$

These crystals were dissolved in 5 cc. of hot water and gave 1.6 gm. of crystals which had the following rotation.

Initial.

No. 358

$$[\alpha]_D^{25} = \frac{-0.15 \times 100}{1 \times 2} = -7.5$$

Nos. 309 and 358 were combined and dissolved in 4 cc. of hot water. There were obtained 1.5 gm. of crystals which had the following rotation in 2.5 per cent HCl solution.

Initial. Final (24 hours).

No. 380

$$[\alpha]_D^{25} = \frac{-0.15 \times 100}{1 \times 2} = -7.5 \qquad [\alpha]_D^{25} = \frac{-0.24 \times 100}{1 \times 2} = -12.0$$

The 1.5 gm. were dissolved again in 1.5 cc. of hot water and gave 1.2 gm. of crystals which had the following rotation in 2.5 per cent HCl solution.

Initial.

No. 381

$$[\alpha]_D^{25} = \frac{-0.15 \times 100}{1 \times 2} = -7.5$$

It crystallized out of 25 per cent alcohol solution in long prismatic needles. Melting point was M. P. = 139°C. (corrected) with decomposition.

Oxidation of Levo-Chondroheptonic Acid by Nitric Acid.

10.0 gm. of substance were dissolved in 70 cc. of water and 30 cc. of 10 per cent hydrochloric acid. 10 gm. of $AgNO_2$ were added and the mixture was allowed to stand over night. 4.0 gm. of $AgNO_2$ and 4 cc. of 10 per cent HCl were then added. An excess of HCl was found, hence $AgNO_3$ was added, filtered, then excess of silver was removed by H_2S. The filtrate was concentrated to 40 cc. under diminished pressure. An equal volume of concentrated HNO_3 was added and heated over flame. It began to react after 2 minutes. The reaction was allowed to proceed 9 minutes and the product was concentrated immediately on a clock glass.

Some crystals, which proved to be oxalic acid, were filtered off and the residue was converted into a calcium salt. The yield was 0.200 gm. The mother liquor was concentrated to very small volume and allowed to crystallize. The yield was 0.800 gm. (No. 191). In 2.5 per cent HCl No. 191 had the following rotation.

$$[\alpha]_D^{25} = \frac{+0.10 \times 100}{1 \times 2} = +5.0$$

Purification of Calcium Salt.

The calcium salts obtained from several experiments were suspended in boiling water and the free acid was liberated by the addition of oxalic acid. The substance was reconverted into calcium salt. The salt obtained in this manner had the following rotation

Initial.

$$[\alpha]_D^{25} = \frac{+0.10 \times 100}{1 \times 2} = +5.0$$

Final.

$$[\alpha]_D^{25} = \frac{+0.07 \times 100}{1 \times 2} = +3.5$$

and gave the following analytical data.

0.0918 gm. substance gave 0.0240 gm. of H_2O, 0.0934 gm. of CO_2, and 0.0238 gm. of ash.

	Calculated for $C_7H_{10}O_7Ca$.	Found.
C	27.52	27.74
H	2.75	2.92
CaO	25.69	25.92

Milder Oxidation of Levo-Chondrosaminoheptonic Acid with Nitric Acid.

10 gm. of heptonic acid, 70 cc. of water, 30 cc. of hydrochloric acid, and 12 gm. of $AgNO_2$ were allowed to stand over night at 10°C. 2 gm. of $AgNO_2$ and 2 cc. of HCl were added in the evening and the following morning the filtrate from Ag_2S was concentrated under diminished pressure without heating to about 40 cc. 10 cc. of this solution were mixed with 10 cc. of concentrated nitric acid and evaporated on clock glass on water bath. The residue was dissolved in a little water and evaporated again. The white residue was taken up in hot water and converted into a calcium salt in the usual way and concentrated under diminished pressure without heating until a precipitate formed (No. 299).

No. 299 had the following rotation in 2.5 per cent HCl solution.

Initial.

$$[\alpha]_D^{25} = \frac{+0.10 \times 100}{1 \times 2} = +5.0$$

On further concentration a further precipitate, No. 300, was obtained.

10 gm. of heptonic acid were deaminized, oxidized, and converted into Ca salt just the same as in the previous experiment. First fraction = 1.7 gm. Second fraction = 0.5 gm.

The calcium salts obtained in this manner were combined and boiled with 500 cc. of water, the insoluble precipitate was filtered off, which had the following rotation in 2.5 per cent HCl solution.

Initial.

$$[\alpha]_D^{25} = \frac{+0.09 \times 100}{1 \times 2} = +4.5$$

Final (24 hours).

$$[\alpha]_D^{25} = \frac{+0.02 \times 100}{1 \times 2} = +1.0$$

It analyzed as follows:

0.1036 gm. of substance gave 0.0288 gm. of H_2O, 0.1032 gm. of CO_2, and 0.0260 gm. of ash.

	Calculated for $C_7H_{12}O_7Ca$.	Found.
C	27.52	27.16
H	2.75	3.11
CaO	25.69	25.09

The filtrate on standing gave a second precipitate (No. 325).

No. 325 had the following rotation in 2.5 per cent HCl solution.

Initial.

$$[\alpha]_D^{25} = \frac{+0.10 \times 100}{1 \times 2} = +5.0$$

Final (24 hours).

$$[\alpha]_D^{25} = \frac{+0.03 \times 100}{1 \times 2} = +1.5$$

It analyzed as follows:

0.0984 gm. of substance gave 0.0258 gm. of H_2O, 0.0998 gm. of CO_2, and 0.0246 gm. of ash.

	Calculated for $C_6H_6O_7Ca$.	Found.
C	27.52	27.65
H	2.75	2.93
CaO	25.69	25.00

The mother liquor of No. 325 was concentrated under diminished pressure until crystallization set in and was then allowed to stand in the cold. A further precipitate was obtained, which had the following rotation in 2.5 per cent HCl solution.

$$[\alpha]_D^{25} = \frac{+0.10 \times 100}{1 \times 2} = +5.0$$

It analyzed as follows:

0.1034 gm. of substance gave 0.0308 gm. of H_2O, 0.1058 gm. of CO_2, and 0.0250 gm. of ash.

	Calculated for $C_6H_6O_7Ca$.	Found.
C	27.52	27.90
H	2.75	3.33
CaO	25.69	24.28

Several samples were combined. The total weight was 8.0 gm. These were suspended in 200 cc. of water and boiled. The first precipitate was removed by filtration.

To the mother liquor neutral lead acetate was added and a precipitate was obtained which was decomposed by H_2S and concentrated under diminished pressure to nearly dryness. The residue was extracted with acetone. The acetone was evaporated to small volume and allowed to stand at room temperature. As it remained syrup it was placed in a vacuum desiccator over H_2SO_4. On long standing it showed a tendency to crystallize. However, crystallization proceeded very slowly, hence it was

transformed into the calcium salt (No. 403). No. 403 was analyzed as follows:

0.1016 gm. of substance gave 0.0278 gm. of H_2O, 0.1050 gm. of CO_2, and 0.0261 gm. of ash.

	Calculated for $C_7H_{14}O_7Ca$.	Found.
C	27.52	28.18
H	2.75	3.05
CaO	25.69	25.68

From the mother liquor a colorless precipitate was obtained (No. 438). No. 438 had the following rotation in 2.5 per cent HCl solution.

Initial. Final (24 hours).

$$[\alpha]_D^{25} = \frac{+0.10 \times 100}{1 \times 2} = +5.0 \qquad [\alpha]_D^{25} = \frac{+0.10 \times 100}{1 \times 2} = +5.0$$

It analyzed as follows:

0.0979 gm. of substance gave 0.0244 gm. of H_2O, 0.0992 gm. of CO_2, and 0.0252 gm. of ash.

	Calculated for $C_7H_{14}O_7Ca$.	Found.
C	27.52	27.63
H	2.75	2.78
CaO	25.69	25.73

Oxidation of Dextro-Chitosaminoheptonic Acid with Nitric Acid.

10 gm. of heptonic acid, 70 cc. of water, 30 cc. of 10 per cent hydrochloric acid, and 12 gm. of $AgNO_2$ were allowed to stand over night in the cold room. Two portions of 2 gm. of $AgNO_2$ and 2 cc. of 10 per cent HCl were added. Further treatment was the same as in the previous experiments.

Three preparations, No. 314, first fraction = 1.25 gm., No. 315, second fraction = 1.05 gm., No. 316, third fraction = 10 gm. of heptonic acid, were treated just the same as previous experiments and 1.75 gm. of calcium salt were obtained.

Purification of the Ca Salt.

All the Ca salts of chitosaminoheptonic acid were combined and boiled with 300 cc. of water. A small part remained insoluble (No. 341). This analyzed as follows:

0.1043 gm. of substance gave 0.0264 gm. of H_2O, 0.1032 gm. of CO_2, and 0.0285 gm. of ash.

	Calculated for $C_5H_6O_5Ca$.	Found.
C	27.52	26.98
H	2.75	2.83
CaO	25.69	27.32

The mother liquor on standing formed a precipitate which had the following rotation in 2.5 per cent HCl solution.

Initial. Final.

$$[\alpha]_D^{25} = \frac{+0.20 \times 100}{1 \times 2} = +10.0 \qquad [\alpha]_D^{25} = \frac{+0.34 \times 100}{1 \times 2} = +17.0$$

It analyzed as follows:

0.0996 gm. of substance gave 0.0290 gm. of H_2O, 0.1062 gm. of CO_2, and 0.0230 gm. of ash.

	Calculated for $C_5H_6O_5Ca$.	Found.
C	27.52	29.07
H	2.75	3.25
CaO	25.69	23.29

From the mother liquor on further standing a further precipitate was obtained which had the following rotation in 2.5 per cent HCl solution.

Initial. Final.

$$[\alpha]_D^{25} = \frac{+0.16 \times 100}{1 \times 2} = +8.0 \qquad [\alpha]_D^{25} = \frac{+0.30 \times 100}{1 \times 2} = +15.0$$

It analyzed as follows:

0.1004 gm. of substance gave 0.0274 gm. of H_2O, 0.1034 gm. of CO_2, and 0.0238 gm. of ash.

	Calculated for $C_5H_6O_5Ca$.	Found.
C	27.52	28.47
H	2.75	3.09
CaO	25.69	23.09

All calcium salts were combined; the total weight was 2.9 gm. They were dissolved in 500 cc. of hot water, 1.0 gm. of oxalic acid was added, and the solution boiled until all the calcium was removed. To the filtrate neutral lead acetate was added and the

precipitate thus formed was decomposed by H_2S and reconverted into the calcium salt (No. 421). It analyzed as follows:

0.1047 gm. of substance gave 0.0276 gm. of H_2O, 0.1060 gm. of CO_2, and 0.0264 gm. of ash.

	Calculated for $C_7H_{12}O_7Ca$.	Found.
C	27.52	27.60
H	2.75	2.94
CaO	25.69	25.21

From the mother liquor of No. 421 on concentration a further precipitate was formed. It was extracted with boiling glacial acetic acid twice, washed with alcohol and ether, and dried. It had the following rotation in 2.5 per cent HCl solution.

Initial.

$$[\alpha]_D^{25} = \frac{+0.17 \times 100}{1 \times 2} = +8.5$$

Final (24 hours).

$$[\alpha]_D^{25} = \frac{+0.29 \times 100}{1 \times 2} = +14.5$$

It analyzed as follows:

0.1104 gm. of substance gave 0.0288 gm. of H_2O, 0.1136 gm. of CO_2, and 0.0280 gm. of ash.

	Calculated for $C_7H_{12}O_7Ca$.	Found.
C	27.52	28.06
H	2.75	2.91
CaO	25.69	25.36

NOTE ON THE LEWIS-BENEDICT METHOD OF BLOOD SUGAR DETERMINATION.

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The experiments recorded in this paper were originally performed for our own information with no purpose of publication. While studying the accuracy of blood sugar estimation we turned our attention to the various components of blood and their effect upon the reaction involved. We found that creatinine behaves with the Lewis-Benedict reagent in a manner similar to that of sugar, and therefore interferes with the quantitative determination of sugar in the blood. We searched for a method whereby the interfering components could be removed from blood preliminary to the sugar analysis, which procedure—we became convinced—was indispensable in working with pathological bloods where the interfering substances may be present in very large quantities. The experiments have been performed at different times during this past year; their perfect agreement is, therefore, the more significant. We are consequently inclined to regard with doubt statements concerning the increased blood sugar level in diseases marked by serious metabolic failure other than diabetes. Thus—"A number of cases of nephritis are reported with hyperglycemias close to 0.2 per cent. In the four cases of interstitial nephritis glucosuria was absent, while mild glucosuria was constantly present in the two cases of parenchymatous nephritis with edema."[1]

We are prompted to publish our observations by reason of the appearance recently of extensive studies by the Lewis-Benedict

[1] Myers, V. C., and Bailey, C. V., The Lewis and Benedict method for the estimation of blood sugar, with some observations obtained in disease, *J. Biol. Chem.*, 1916, xxiv, 147.

method of the blood sugar content in various pathological states[2, 3] which have not reckoned with the effect the increased blood creatinine may have had on the blood sugar values, as is shown by the following quotation—"In the last stages of nephritis, when the patient is in uremia, the blood sugar will be found very high, often equalling the severe stages of diabetes. Other important metabolic constituents of the blood will be found correspondingly increased, presenting a picture of complete metabolic failure."[3]

In summing up our experiments, they fall into four groups according to the material used, but they all agree in showing the influence of creatinine upon the intensity of the color produced by a definite quantity of glucose with picric acid.

Table I records a series of observations with solutions containing 100 mg. of glucose in 100 cc. of water, to which various quantities of creatinine were added to produce a range of concentrations encountered in both normal and abnormal bloods.

TABLE I.

Solution.	Creatinine per 100 cc. added.	Glucose per 100 cc.		Difference.	
		Expected.	Found.		
	mg.	*mg.*	*mg.*	*mg.*	*per cent*
1. Glucose	None.	—	100	—	—
2. "	2	100	110	10	10
3. "	5	100	120	20	20
4. "	6	100	130	30	30
1. "	None.	—	100	—	—
2. "	5	100	120	20	20
3. "	10	100	140	40	40
4. "	15	100	150	50	50
5. "	20	100	160	60	60
6. "	25	100	180	80	80
7. "	30	100	210	110	110

[2] Gettler, A. O., and St. George, A. V., The value of modern blood chemistry to the clinician, *J. Am. Med. Assn.*, 1918, lxxv, 2033.

[3] Williams, J. R., and Humphreys, E. M., Clinical significance of blood sugar in nephritis and other diseases, *Arch. Int. Med.*, 1919, xxiii, 537.

It may not be amiss to point out here that all the determinations were carried out strictly in accordance with the Lewis-Benedict method. The picric-picrate reagent used was of the proper acidity, all the test-tubes of the same series were boiled simultaneously and for the same length of time, and the color comparisons were made with a Leitz-Duboscq colorimeter.

Another series of experiments recorded in Table II was made with solutions containing definite proportions of glucose and creatinine, and protein in the form either of egg white, egg albumin, or dialyzed blood serum.

TABLE II.

Solution.	Creatinine per 100 cc. added.	Glucose per 100 cc.		Difference.	
		Expected.	Found.		
	mg.	*mg.*	*mg.*	*mg.*	*per cent*
1. Egg white in 0.9 per cent NaCl + glucose	None.	—	210	—	—
2. Egg white in 0.9 per cent NaCl + glucose	1	210	210	—	—
3. Egg white in 0.9 per cent NaCl + glucose	2.5	210	220	10	5
4. Egg white in 0.9 per cent NaCl + glucose	5	210	260	50	24
1. Egg albumin in 0.9 per cent NaCl + glucose	None.	—	130	—	—
2. Egg albumin in 0.9 per cent NaCl + glucose	10	130	190	60	46
3. Egg albumin in 0.9 per cent NaCl + glucose	20	130	230	100	77
4. Egg albumin in 0.9 per cent NaCl + glucose	30	130	260	130	100
1. Dialyzed blood serum + glucose	None.	—	130	—	—
2. " " " + "	1	130	130	—	—
3. " " " + "	2.5	130	140	10	8
4. " " " + "	5	130	160	30	23

The results recorded in Table III were obtained with sheep's blood to which definite amounts of creatinine had been added.

TABLE III.

Blood.	Creatinine per 100 cc. added.	Glucose per 100 cc.		Difference.	
		Expected.	Found.		
	mg.	*mg.*	*mg.*	*mg.*	*per cent*
1. Fresh sheep's blood	None.	—	80	—	—
2. " " "	5	80	90	10	13
3. " " "	10	80	100	20	25
4. " " "	15	80	110	30	37
1. Sheep's blood, 2 days old	None.	—	50	—	—
2. " " 2 " "	20	50	140	90	180
3. " " 2 " "	30	50	180	130	260
4. " " 2 " "	40	50	230	180	460

TABLE IV.

Blood.	Creatinine per 100 cc. added.	Glucose per 100 cc.		Difference.	
		Expected.	Found.		
	mg.	*mg.*	*mg.*	*mg.*	*per cent*
1. Mr. X	None.	—	150	—	—
2. "	5	150	160	10	6.6
3. "	10	150	180	30	20
1. Mr. T.	None.	—	120	—	—
2. "	10	120	160	40	33
1. Mr. T.	None.	—	140	—	—
2. "	10	140	180	40	29
1. Mrs. W.	None.	—	160	—	—
2. "	10	160	220	60	38
3. "	20	160	270	110	70
4. "	30	160	310	150	94
1. Mr. G.	None.	—	185	—	—
2. "	10	185	240	55	30
3. "	20	185	273	88	47.5
4. "	30	185	316	131	70.9
5. "	40	185	375	190	102.7

Table IV contains our data with human blood on which sugar determinations were made by the Lewis-Benedict method. The creatinine added to the different samples of blood ranged from 5 to 40 mg. per 100 cc. of blood.

SUMMARY AND CONCLUSION.

Reviewing all the data presented in this paper one cannot escape the conclusion that the presence of creatinine increases the blood sugar value as determined by the Lewis-Benedict method. It is true that concentrations less than 2 mg. of creatinine per 100 cc. have no effect upon the accuracy of the reaction, but with greater concentrations the error in the sugar analysis increases progressively. It is probable, therefore, that the Lewis-Benedict method is applicable to normal bloods where the creatinine concentration is about 1 to 3 mg. per 100 cc., but under pathological conditions when the creatinine level may rise to 10 to 40 mg. per 100 cc. the sugar analysis by the Lewis-Benedict method loses its value as a quantitative procedure.[4]

[4] An investigation of the blood sugar in disease is now under way. The object is to collect data that will be free from the criticism raised in this paper against the blood sugar determinations by the Lewis-Benedict method.

ISOLATION OF THE IODINE COMPOUND WHICH OCCURS IN THE THYROID.*

FIRST PAPER.

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Despite the many years of investigation of the thyroid, some of the most fundamental problems concerning this ductless gland are still the subject of speculation and controversy, and the certain progress which follows quantitative knowledge has not yet materialized. Since quantitative values cannot be obtained by clinical observations alone, and since pathologic studies of the glands cannot of themselves solve the function of the thyroid, it becomes apparent that nothing short of the actual isolation in pure crystalline form of the chemical substance or substances within the gland, which are responsible for its activity, can furnish the necessary knowledge with which to gain quantitative relationship. Having accomplished the isolation in pure crystalline form of the active agents within the gland, quantitative results may be obtained and ultimate solution of the thyroid problem is within the power of the investigator.

Investigation of the Chemical Constituents of the Gland, with Isolation of the Iodine-Containing Compound in Pure Crystalline Form.

Starting with fresh and desiccated thyroid the present investigation was at first concerned with the diffusibility of the iodine-containing compound. Iodine is not dialyzable from the thyroid proteins, and will withstand rather severe chemical treatment

*No historical review of the chemical investigation of the thyroid is given in this paper. The reader is referred to the attached Bibliography.

and still be undialyzable through a collodion sack in running water. In order to determine the stability of the iodine compound, various hydrolytic processes were applied to the thyroid proteins. Among others the alcoholic alkaline hydrolysis used by Vaughan was tried and, with some slight modifications, was found to produce a deep seated hydrolysis without breaking off iodine from its organic combination. Furthermore, the hydrolysis so alters the iodine-containing compounds that about 70 per cent of the total iodine is dialyzable. Another change brought about by the hydrolysis is the solubility of the iodine compound in acid. About 50 per cent of the total iodine contained in the hydrolyzed products is soluble in acids, and 50 per cent is insoluble. The iodine in the acid-insoluble portion is to a large extent not dialyzable. The presence of iodine in organic combination and in non-dialyzable form was encouraging evidence of the stability of the iodine compound and invited further investigation.

The physical and chemical properties of the acid-insoluble group of hydrolyzed constituents will be described somewhat in detail, since this will bring out the chemical problems involved and some of the difficulties encountered.

An alkaline solution of the acid-insoluble constituents is dark brown, almost black, with a green fluorescence, and shows a Tyndall phenomenon with a beam of light. The non-diffusibility, fluorescence, and Tyndall phenomenon show that the solution is one of colloidal nature. Among the acid-insoluble constituents are fatty acids resulting from the original fat in the desiccated thyroid, and sulfur, which results from the decomposition of cystine. The solution has no characteristic odor other than a general fatty smell. The first step in the method of separating the iodine compound is to dry the acid-insoluble constituents, mix with infusorial earth, and extract with petroleum ether to remove fatty acids and sulfur.

Later it was found that fresh thyroid glands could be substituted for desiccated thyroid as a source of material, and that hydrolysis in alcohol was not necessary. Hydrolysis of the proteins may be carried out by use of aqueous sodium hydroxide alone, and the length of the time can be reduced to 24 hours. A quantitative separation of all fats as the sodium soap may be

effected, and a perfectly clear alkaline filtrate of the hydrolyzed thyroid proteins containing practically the entire iodine content of the gland is obtained. On acidification of this solution, a fine flocculent precipitate separates. If this precipitate is filtered off and dried, it is found to contain approximately 0.1 per cent of the total weight of the fresh glands used. It contains, on the average, 26 per cent of the total iodine. The total iodine in the gland, therefore, is divided by aqueous alkaline hydrolysis into acid-soluble and acid-insoluble compounds. Approximately three-fourths of the total iodine-containing compounds are soluble in acid and only one-fourth insoluble. This proportion is remarkably constant during different times of the year, and in different samples of thyroid from different species. The significance of the figure is not entirely clear, but in all probability it represents the equilibrium existing within the glands between the completed iodine compound which possesses physiologic activity and the materials which are used by the gland in the building up of the substance. Physiologically tested, the acid-soluble hydrolyzed constituents are inactive, and the small portion which is precipitated by acid possseses the entire physiologic activity of the gland.

The solubility of the acid-insoluble iodine compounds in organic solvents varies, depending on the solvent used, the presence of water, whether or not acid is present, and the temperature. About 10 per cent of the total iodine is soluble in ethyl ether, but only a small amount is soluble in petroleum ether.

If desiccated thyroid has been used as a source of material the dry acid-insoluble products freed from fatty acids by extraction with petroleum ether are dissolved in sodium hydroxide and again acidified. If the aqueous hydrolysis has been used the precipitate obtained by acidifying the alkaline solution of hydrolyzed products is dissolved in sodium hydroxide and reprecipitated with acid. This precipitate is heavy, flocculent, and amorphous, and, when heated above 40–50°C. in aqueous solution, turns to a black, tarry mass. On cooling and drying it is found to be brittle and may be broken into a fine, dry, almost black powder. The dry powder is soluble in acid ethyl alcohol and aqueous solutions of sodium, potassium, and ammonium hydroxides and carbonates. It is in part precipitated from acid

alcohol by the addition of sodium carbonate, the portion precipitated being a sticky, black, tarry mass. Most of the iodine-containing compounds are soluble in alcohol in the presence of sodium carbonate, so that this is a valuable aid in the gross separation of the black, tarry impurities from the constituents which contain iodine. However, some of the iodine is carried down by the sodium carbonate precipitation, showing that the iodine compound, although probably the same throughout all the different precipitates, is attached to various groups of compounds which possess different solubilities.

If barium hydroxide is added to the alcohol solution after removal of the sodium carbonate precipitate, practically all the rest of the dark-colored compounds are precipitated by the barium. This precipitate carries down varying percentages of iodine, depending on conditions. If the solution is made acid with hydrochloric acid before the addition of barium, a very high percentage of the iodine may be precipitated. If the solution is made alkaline with sodium hydroxide, a very small percentage may be carried down. Between these two limits the amount removed by the barium depends on the acidity and the temperature. Since the alcohol solution remaining after precipitation with barium is light straw-colored, the alcohol-barium-soluble constituents to which the iodine compound is attached are almost colorless.

Similar separations may be produced in aqueous solution. If the dark brown alkaline solution of the hydrolyzed products is acidified, almost all the color and iodine compounds are precipitated. Barium, calcium, and magnesium salts, added to an alkaline aqueous solution of the acid-insoluble hydrolyzed constituents, precipitate practically all the dark-colored compounds, leaving a very light-colored solution. This precipitation divides the iodine present; about one-half is precipitated by the alkaline earths and one-half is soluble.

These precipitation and solubility properties clearly show the nature of the material with which one has to work. It is a mixture of compounds in colloidal form, and the problem is not only one of separating a mechanical mixture. It was necessary to gain a clear insight into the chemical nature of the iodine compound and the other constituents before the separation of the two could be realized.

The most striking property of the acid-insoluble group of hydrolyzed products is their acidic nature. They may be dissolved in alkali and reprecipitated by acid without appreciable loss of iodine.

Many attempts were made to separate the iodine compound by its solubility and precipitation properties with various reagents. No specific precipitant was found. No difference in solubility was found which could be used to effect a separation. After many attempts to separate the iodine compound had failed, it became apparent that the compound was not present in free form, but was still firmly bound to some unknown substances.

Experiments showed that iodine was not broken off when dissolved in moderately strong sodium hydroxide. In the hope that heating in aqueous sodium hydroxide might produce a further hydrolysis, the acid-insoluble constituents of the alkaline-alcoholic hydrolysis were heated several hours in 5 per cent sodium hydroxide. It was then found that treatment with sodium hydroxide, followed by precipitation with an acid, will not effect a separation of the iodine compound from the black colloidal impurities. The solubility of the compounds present which do not contain iodine closely parallels the solubility of the iodine-containing compound, and, as far as alkali and acid are concerned, no appreciable separation can be brought about by alternate treatment with each.

Since the addition of a soluble barium salt to an alkaline solution of the acid-insoluble products of hydrolysis precipitates practically all the brown impurities and does not carry down all the iodine, this separation was investigated to see whether the percentage of iodine in the acid-insoluble constituents could thereby be increased. The acid-insoluble constituents were dissolved in dilute sodium hydroxide, and barium hydroxide was added. The solution was heated in a nickel crucible at 100°C. for 18 hours. This treatment produced a precipitation of the brown compounds, giving a light yellow filtrate of the barium-soluble constituents. Determination of the iodine content of the filtrate showed that about 50 per cent of the iodine present had been precipitated from the alkaline solution by barium hydroxide, and 50 per cent remained soluble. Sodium sulfate was added to the water solution containing the barium-soluble con-

stituents, and the barium was removed as sulfate. The solution was then acidified, and a copious precipitate obtained which still retained iodine. The precipitate was filtered off and dried. Analysis showed that the percentage of iodine had been very materially increased by this treatment. The iodine in the starting material amounted to about 5 per cent; after treatment with barium, as outlined above, and reprecipitation with an acid, the iodine was found to be about 15 per cent.

The portion of the starting material which had formed a dark, flocculent precipitate with barium hydroxide was dissolved in sodium hydroxide and sodium sulfate. The barium sulfate was removed and the solution was acidified. A precipitate formed on the addition of the acid and when this was filtered off and dried it too was found to contain an increased percentage of iodine. By treatment with barium hydroxide the percentage of iodine contained in the hydrolyzed constituents of the thyroid proteins had been increased.

As this was the only material advance which had been made toward isolation of the iodine-containing compound, it was decided to investigate more fully the reactions involved between barium and the hydrolyzed products of the thyroid proteins. The two precipitates were dissolved in separate solutions of sodium hydroxide, barium hydroxide was added to each, and the solutions were heated in nickel crucibles for 18 hours at 100°C. The insoluble portions were filtered off, the barium was removed as outlined above, the barium-soluble constituents of each crucible were combined and again acidified, the precipitate was filtered off and dried, and analysis showed that the percentage of iodine present had increased in the barium-soluble portion to 26 per cent. With many misgivings the precipitation with barium and heating in a nickel crucible were repeated still another time. The percentage of iodine in the precipitate obtained was 33 per cent. Still another treatment yielded a product containing 42 per cent of iodine. The amount of material now consisted of less than 200 mg. The success of the method, however, called for still further treatment. It was carried out in the same manner, and a precipitate containing 47.3 per cent of iodine was obtained. The character of the precipitate had changed and the color of the solution had almost disappeared. It seemed

probable that some other method of purification could now be applied to the preparation containing 47.3 per cent iodine.

The material was dissolved in 95 per cent alcohol; solution was complete. The alcohol was evaporated on a water bath to a small volume, in the hope that some crystals might separate. By a chance, however, the dish containing the alcohol evaporated to dryness, and the dried precipitate was heated for about 1 hour after the alcohol had been driven off. It was thought that on the addition of more alcohol the white powder on the bottom of the dish would again be dissolved, and, possibly, crystals might still separate. More alcohol was therefore added, but a white incrustation on the bottom of the dish was insoluble in alcohol. I believed that this treatment had effected a further separation, that the iodine-containing compound was redissolved in the alcohol, and that the white incrustation represented impurities. It was filtered off. The weight of this white powder was 18.6 mg. It was dissolved in sodium hydroxide, and a portion of it was used to determine iodine, when it was found that the iodine amounted to 60 per cent. The white incrustation was, therefore, not an impurity, but the iodine compound itself. Its solubility in alcohol had been changed by heating the residue left after evaporation of the alcohol. More of the acid-insoluble constituents resulting from hydrolysis of the thyroid proteins were treated in precisely the same manner, and about 200 mg. of the white residue were obtained. When this was dissolved in aqueous sodium hydroxide, precipitated by adding sulfuric acid and boiling, it was converted into fine white microscopic crystals. For reasons which will be given this iodine compound has been named "thyroxin," and it will be referred to by that name throughout the remainder of this article.

As the yield of thyroxin depended on the amount of desiccated thyroid which could be treated at any one time, it was decided to enlarge our facilities for the hydrolysis of desiccated thyroid, which, up to this time, had been carried out in glass flasks. An 11 gallon galvanized iron tank was constructed, which could take care of 500 gm. of desiccated thyroid at one time. The acid-insoluble constituents were obtained as before. They were dissolved in dilute sodium hydroxide, barium hydroxide was added, and the steps outlined above were carried out. No crystals of thyroxin were obtained.

The work from this point will be presented in a logical rather than a chronological order, as many months were spent in elucidating the factors which prevented the isolation of more crystals.

After working for 14 months in an endeavor to repeat the first isolation of the crystals, it was found that there are five conditions which influence the isolation of thyroxin in pure crystalline form.

1. Effect of Temperature on Precipitation with an Acid.—When the partially purified, iodine-containing constituents are precipitated by an acid, the precipitate is flocculent and amorphous. If this is heated to 40–50°C., it assumes a fine, granular form which can be filtered and washed very readily. This procedure was followed because of the facility of handling the precipitate. Warming of the acid solution does not break off any iodine in inorganic form. If, however, this precipitate, which was prepared by warming the solution after the addition of the acid, is dissolved in alkali and heated with barium hydroxide, and the solution is again acidified, some iodine in the inorganic form may be found in the solution. If the precipitation by an acid is carried out in the cold, subsequent heating with barium hydroxide and reprecipitation does not break off iodine in the inorganic form.[1]

Since only one stage of this treatment could be carried out in 1 day, 24 hours intervened between the first acidification and warming of the solution and the second precipitation, after which iodine was demonstrated in the inorganic form in the filtrate. This time interval formed a convenient screen behind which the deleterious action of heat remained hidden for many months. This influence of temperature on acidification was one of the most important causes for the failure to separate more crystals. Fearing that a concentrated sodium hydroxide solution would destroy the iodine compound, we had been neutralizing the alkali which was used to effect the primary hydrolysis with sulfuric acid before evaporating off the alcohol. As the amount of free alkali remaining in the alcohol was variable, depending on the amount of ammonia which had been evolved and the amount of

[1] Inorganic iodine has been tested for during this investigation by acidifying the solution with hydrochloric acid, adding starch and a few drops of sodium nitrite, producing a blue ring if inorganic iodine is present.

water and carbonate in the alkali, it frequently happened that slight excess of acid was added, so that the alcohol was evaporated off in the presence of a small amount of acid. This we finally showed has a very destructive action on partially purified thyroxin. In this connection it seems probable that the lack of physiologic activity of iodothyrin is explained by this action of acid on the iodine-containing compound. The iodine may not be broken off by treatment with acid, but the chemical properties, and undoubtedly the nucleus to which the iodine is attached, are altered so that the compound loses its physiologic activity.

2. Effect of Heating the Alkaline Hydrolysis Solution in the Presence of Metal.—At first the hydrolysis was carried out in glass flasks. When an 11 gallon galvanized iron tank was substituted, the hydrolysis was carried out apparently just the same, but it was found that with the metal the hydrolysis in an alkaline solution resulted in the breaking off of the iodine in the inorganic form. Investigation showed that in alkaline solution, iron, zinc, copper, tin, lead, German silver, and, in fact, all metals except nickel and the heavy metals, gold, silver, and platinum, break off iodine from its organic combination. A heavily enameled, cast iron kettle was found to give a satisfactory surface for a container in which to carry out the primary hydrolysis with sodium hydroxide in alcohol. Later this was replaced by a nickel kettle.

3. Effect of Carbon Dioxide.—During the first separation of crystals carbon dioxide was neither employed directly nor was its effect excluded from the various steps in the process. After many months of failure to separate more of the iodine compound in crystalline form it was found that carbon dioxide plays an important rôle in the separation of thyroxin from the impurities. By chance an alkaline solution containing a partially purified preparation of thyroxin mixed with a large amount of impurities was precipitated with carbon dioxide instead of sulfuric acid. The precipitate was filtered on a Buchner funnel and washed with distilled water. Instead of the distilled water running through lighter colored than the first filtrate, it was almost black (Fig. 1). Investigation showed that the black impurities were insoluble in water saturated with carbon dioxide but were soluble in distilled water. Even after the effect of the carbon dioxide on the separation of thyroxin was discovered, its explanation was, for many months, obscure.

4. Effect of Temperature during Treatment of a Solution with Carbon Dioxide.—After it was found that carbon dioxide had a very important action which allowed thyroxin to be isolated in crystalline form, carbon dioxide was added to the various solutions, first in the cold, and then it was added to the solutions warmed to various temperatures up to 100°C. It was found that with the preparations which were contaminated with a large

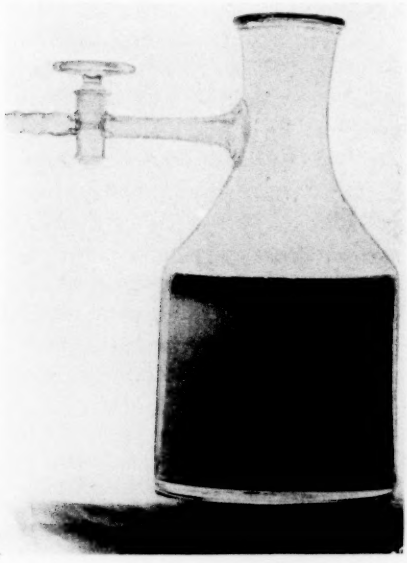


FIG. 1. Suction flask showing the color change which led to the investigation of the effect of carbon dioxide on the separation of thyroxin.

amount of impurities, the passing of carbon dioxide into a solution above 50–60°C. resulted in breaking off iodine. This was confirmed many times, and although for a long time unexplained, the solutions were always cooled before treatment with carbon dioxide.

5. Effect of Different Samples of Desiccated Thyroid.—The fifth point is one beyond control, and was found to rest on the condition of the desiccated thyroid employed. The thyroxin content of different samples of desiccated thyroid varies as much as 400

per cent. It appears probable that bacterial or other decomposition so alters the proteins in some samples that it is impossible to separate any of the iodine compound.

These five factors finally became apparent after a consideration of all the results obtained over a course of 2 years investigation. It is remarkable that all these factors were unconsciously controlled during the first purification, especially as it took many months to find out that there were so many separate and distinct influences at work causing the destruction of the compound.

The investigation of the chemical constituents of the thyroid was begun by the writer in September, 1910. The use of barium salts to effect a separation was tried in November, 1914, and thyroxin was first isolated in December, 1914. Although over 100 mg. were isolated at that time, it was not until February, 1916, that the effect of carbon dioxide was established and more of the substance was obtained. During the summer of 1916 several grams of thyroxin were separated, and by May, 1917, over 7 gm. were available for its chemical identification. The empirical and structural formulas were determined during the summer of 1917. In December, 1917, Mr. Osterberg succeeded in synthesizing a small amount of thyroxin. The synthesis was repeated and the structural formula confirmed in April, 1919. Up to the present time about 33 gm. of the compound have been separated from 6,550 pounds of fresh thyroid material which has been made up almost entirely of hog thyroid (Fig. 2). The method may be briefly stated as follows.

The fresh thyroid glands are hydrolyzed in 5 per cent sodium hydroxide. The fats are removed by rendering the sodium soap insoluble, and the clear alkaline filtrate is cooled and acidified. The acid-insoluble constituents containing practically 100 per cent of the thyroxin present are filtered off. This material is redissolved in sodium hydroxide and reprecipitated, using hydrochloric acid. The precipitate is now air-dried and is dissolved in 95 per cent alcohol. The excess hydrochloric acid which remains in the air-dried precipitate is neutralized with sodium hydroxide until it is almost neutral to moistened blue litmus paper. A heavy, black, tarry precipitate forms, which may be removed by filtration. The alcoholic filtrate is treated with barium hydroxide by adding a hot, very concentrated aqueous solution of the

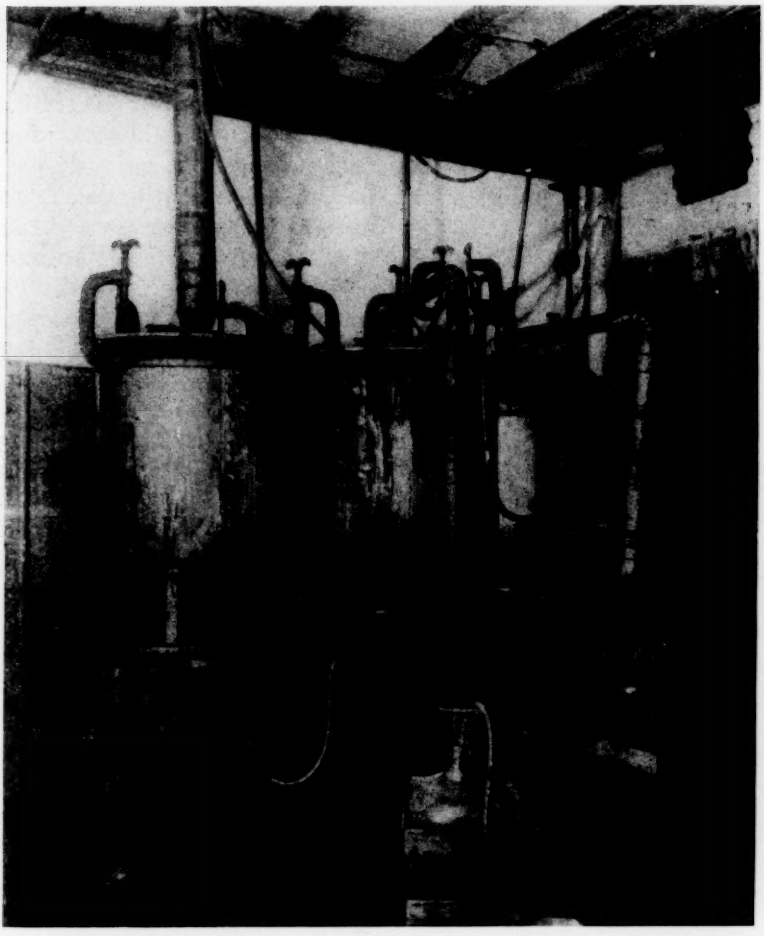
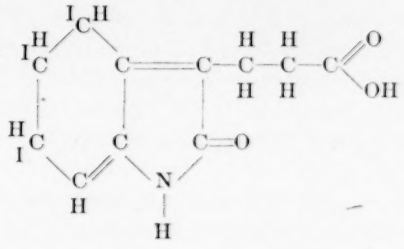


FIG. 2. The three tanks in which 6,550 pounds of hog thyroid glands were treated for the isolation of thyroxin.

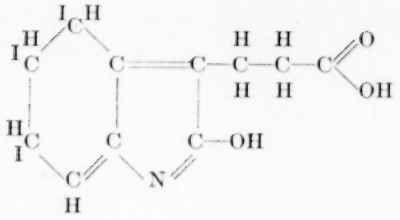
hydroxide to the alcohol, and refluxing. The treatment with barium removes some heavy dark impurities. A small amount of sodium hydroxide is added to the filtrate, and carbon dioxide is passed through the solution. The barium and sodium carbonate are removed by filtration, and the alcohol is distilled.

The last traces of alcohol are removed by heating in an evaporating dish. The aqueous residue is now acidified with hydrochloric acid. The precipitate is dissolved in alkaline alcohol, carbon dioxide is passed through the solution, the precipitated sodium carbonate is removed, and the alcohol is evaporated. The last traces of alcohol are removed by heating on a water bath and the solution is allowed to stand. The monosodium salt of thyroxin will separate at this point. The yield is not quantitative, and it must be further purified by dissolving in alkaline alcohol, passing in carbon dioxide, distilling the alcohol, and allowing the monosodium salt to crystallize a second time. This may then be precipitated from an alkaline alcoholic solution by the addition of acetic acid. Resolution in alkaline alcohol and precipitation with acetic acid for five or six times removes the impurities and will yield thyroxin containing the theoretical percentage of iodine. A considerable percentage of the total iodine present is carried down in the neutral alcohol solution by the barium, and another portion is held in solution when the monosodium salt separates, but it is not practicable to try to separate the thyroxin from these precipitates as the yield is very small. Physiologically they possess the same activity as thyroxin when administered according to the iodine content.

After isolating about 7 gm. of thyroxin in the manner described, its empirical and structural formulas were determined and the substance was shown to be 4, 5, 6 tri-hydro-4, 5, 6 tri-iodo,-2 oxy,-beta indolepropionic acid. Thyroxin exists in three forms: (1) the keto form with the carbonyl group adjacent to the imino,



(2) a tautomeric enol form of this with an alpha hydroxy group and double-bonded nitrogen with no hydrogen attached to the imino,



and (3) a form in which there is an open-ring structure, the elements of water entering between the imino and the carbonyl with the formation of an amino and a carboxyl group. A consideration of the isolation of thyroxin after its structural formula had been determined explains the chemical reactions involved in the purification and isolation of the substance.

A Consideration of the Reactions Involved in the Isolation of Thyroxin.

Thyroxin was first separated by following a method of treatment which was found to increase progressively its iodine content, and for the isolation of much of the material so far prepared this same method was followed without any light being thrown on the exact nature of the chemical reactions involved. The use of barium salts for the separation of thyroxin was first tried because of their ability to precipitate the dark-colored impurities from an alkaline solution of the hydrolyzed thyroid proteins. With the solutions of hydrolyzed proteins at first used, about 50 per cent of the total iodine could be precipitated by barium hydroxide. When the barium-soluble constituents were precipitated by an acid and redissolved in sodium hydroxide or carbonate, barium salts were found to precipitate some of the compounds which had previously been soluble in the presence of barium. This in time led to the discovery that the solubility of thyroxin in the presence of barium hydroxide was a test of its purity.

Partially purified thyroxin which is soluble in barium hydroxide is also soluble in sodium carbonate and in alcohol. Pure thyroxin is insoluble in these reagents. The process of purification rested essentially on the repeated treatments of the impure preparations of thyroxin with barium hydroxide and the recovery

of the barium-soluble compounds by precipitation with an acid. The barium-insoluble constituents were recovered as sodium salts after decomposition with sodium sulfate and then precipitated with an acid.

After six or eight such barium treatments it was found that an increasing percentage of the total iodine was insoluble in barium hydroxide and finally it was shown that thyroxin could not be separated in crystal form from alcohol so long as it was soluble in barium hydroxide. Treatment with barium hydroxide influenced the separation of thyroxin in four different ways: (1) it effected a separation between the two forms of thyroxin, one barium-soluble, the other barium-insoluble; (2) it caused the destruction of certain of the impurities which were present; (3) it precipitated certain impurities from the solution thereby effecting a separation; and (4) it carried down mechanically, when it was precipitated as barium sulfate, the dark-colored colloidal impurities which had almost identical solubilities as thyroxin itself and which were not removed by any other means.

The chain of events which led to the explanation of the reactions involved in the separation of thyroxin from the other compounds contained in the acid-insoluble products of the alkaline hydrolysis and the conversion of barium-soluble thyroxin into barium-insoluble thyroxin was as follows.

It was found that derivatives attached to the imino group render thyroxin soluble in alcohol. This was found true of the acetyl, the formyl, the ureide, the sulfate, and the hydrochloride. It was also found that derivatives attached to the imino group do not form crystalline disilver salts.

When thyroxin is partially purified, so that it contains from 30 to 50 per cent of iodine, it is soluble in alcohol and it does not form a crystalline silver salt. These reactions suggested that the difficulty in purification arose from the fact that some derivative was attached to the imino group, which rendered the compound soluble in alcohol and prevented its precipitation with silver. Since it had already been found that the sodium salts of the acetyl, the formyl, and other derivatives on the imino were readily thrown out of solution in crystalline form by increasing the amount of sodium hydroxide present, attempts were made to determine the group attached to the imino in partially purified

thyroxin by precipitating its sodium salt with a high concentration of sodium hydroxide. Partially purified barium-soluble thyroxin containing about 40 per cent of iodine was therefore dissolved in sodium hydroxide, and a solution of 30 per cent sodium hydroxide was added to this. As had been hoped, the addition of the stronger alkali soon produced a cloudy precipitate which did not settle but remained suspended, due to the high specific gravity of the solution. This was centrifugalized, the supernatant liquid contained most of the yellow impurities, and the precipitate remained as a firm felt on the bottom of the tube. It was dissolved in distilled water, sodium hydroxide was again added, the precipitate again formed, but this time it separated in more distinct particles and the solution was less turbid. It was centrifugalized, the supernatant liquid was still yellow, but showed much less color than the first solution. The residue in the bottom of the tube was again dissolved in distilled water, sodium hydroxide was added, and this time the precipitate assumed a still different form, coming down in distinct separate particles, practically white, and the solution was almost colorless. These particles were examined under the microscope. They were the typical disodium salt of pure thyroxin itself, and not of a derivative. They were centrifugalized from the alkali, dissolved in alcohol, and precipitated by the addition of acetic acid, when they were recovered as pure crystalline thyroxin.

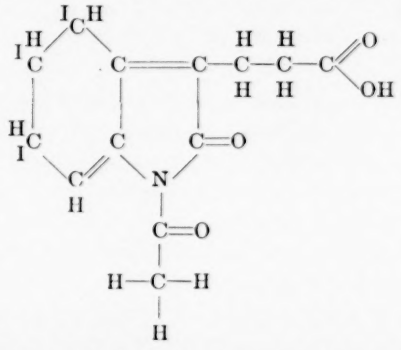
The substitution of sodium chloride for sodium hydroxide permitted the solutions to be filtered instead of centrifugalized, and it was then found that sodium chloride precipitated the disodium salt of thyroxin from an alkaline solution even better than sodium hydroxide. Although this sample of thyroxin had been completely soluble in barium hydroxide, it was now insoluble in such a solution. As this change in solubility could not have been brought about by the hydrolysis of a derivative attached to the imino group, it was apparent that solubility in barium hydroxide depends on the presence of certain impurities.

Investigation of the impurities which were separated from thyroxin by salting out the crystals from the alkaline solution showed that they are soluble in ether, that they are acidic in nature, and contain indole derivatives which give the pine-splinter reaction after fusion in caustic alkali. If pure crystalline

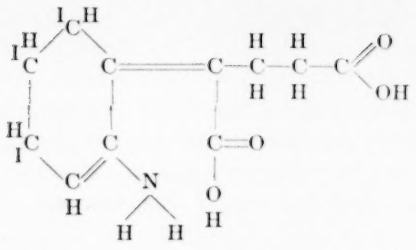
thyroxin is dissolved in dilute sodium hydroxide and some of these indole derivatives are added to the solution, there appears to be an immediate reaction between thyroxin and these impurities which completely alters the chemical properties of thyroxin. The presence of these impurities renders thyroxin soluble in barium hydroxide, and instead of separating with sodium chloride as the crystalline disodium salt, it is thrown out of solution as an oily tar.

Since it was shown that this great alteration in the chemical properties of thyroxin could be brought about by the presence of certain indole derivatives of acidic nature, and since the change was not due to the hydrolysis of a derivative attached to the imino group, the first explanation was that the imino group was rendered more reactive by the presence of the impurities and that a salt formation occurred similar to the sulfate or hydrochloride, the acidic group being attached to the imino nitrogen. However, it was difficult to explain all the reactions by such a change.

The first proof of the chemical reactions involved was obtained after preparation and analysis of the acetyl derivative. In the acetyl derivative of thyroxin, only one acid group is left which can react with a metal, therefore, a mono-metal derivative



should be obtained. Analysis of the silver salt of the acetyl showed that apparently two atoms of silver had added to the acetyl. The simplest explanation for this would be the opening of the pyrrole ring between the imino and carbonyl groups with the formation of the second free carboxyl group.



Further investigations showed that this is most probably true and that a similar opening of the ring occurs in thyroxin. The relation between the two forms is precisely the same as the relation between creatinine and creatine. The opening of the ring increases the acidic properties of thyroxin and renders it soluble in sodium carbonate, barium hydroxide, and alcohol. It prevents the formation of a crystalline insoluble disilver salt and, in fact, all the changed properties of thyroxin are adequately explained by this reaction.

Analysis shows that the iodine content of barium-soluble thyroxin may be as high as 58 or even 60 per cent. This iodine content excludes the possibility of any group being attached to the imino, having a higher molecular weight than formic acid. Furthermore, barium-soluble thyroxin can be changed and separated in pure crystalline form, insoluble in barium hydroxide, merely by allowing an aqueous sodium carbonate solution to stand several weeks. Under these conditions there is a slow closing of the ring with the formation of the mono-salt which is only slightly soluble. It seems most probable that the impurities present do not react in a stoichiometric relation with all the thyroxin, but that the presence of even a small percentage of the impurities introduces the factor of time and greatly delays the rate at which the ring closes.

Sufficient proof that a group is not attached to the imino group of partially purified thyroxin which is soluble in barium hydroxide, alcohol, and sodium carbonate is furnished by the fact that it is impossible to hydrolyze the acetyl from the imino group without disruption of the molecule. This also applies to all derivatives so far studied in which a group is attached to the nitrogen. Hydrolysis does not remove the group but destroys

the integrity of the molecule. Therefore, the reactions occurring during purification can not be the removal of a group attached to the imino. In the body it appears probable that thyroxin exists in the open-ring form with an amino and two carboxyl groups. The problem then, in isolation is to establish conditions favorable for the closing of the pyrrole ring. The open-ring form of thyroxin will not crystallize and although it is but slightly soluble in acid, its solubility in carbonates, barium and calcium hydroxide, alcohol, and pyridine are in striking contrast to the closed-ring form which is insoluble in all these reagents. In the presence of certain impurities it appears very difficult, sometimes impossible, to close the ring and thereby separate thyroxin. The mere presence of these impurities is sufficient to open the ring if pure crystalline thyroxin is added to a solution of such impurities.

The exact nature of these impurities is still unknown, but they are among the hydrolyzed products of the thyroid proteins and contain the indole nucleus. Animo-acids from gelatin do not cause thyroxin to exist in the open-ring form. The difficulties encountered in separating the closed-ring form of thyroxin from any solution depends on the ratio between the amount of thyroxin present and the amount of impurities present. Thyroxin appears to carry down either chemically or mechanically these impurities so that solution in alkali and precipitation with acid do not effect a separation. The best conditions so far found for the closing of the ring and the separation of thyroxin are obtained by solution of thyroxin in sodium carbonate. Under these conditions the ring slowly closes and the monosodium salt, which is only slightly soluble, deposits in crystalline form on the bottom of the flask.

SUMMARY.

The chemical reactions involved in the five conditions, which influence the isolation of thyroxin, may be summarized as follows.

1. Effect of Temperature on Precipitation with an Acid.—The chemical reactions involved in the acidification with an acid were very obscure until the acetyl derivative was prepared. It was found that when an acid suspension of the acetyl is neutralized with sodium hydroxide, at the neutral point, iodine breaks off

from the acetyl derivative, and the liberation of iodine may be shown to be a matter of oxidation and reduction. Thyroxin is far less susceptible, and an acid suspension of thyroxin may be neutralized with an alkali without any such liberation of iodine occurring. When, however, an impure preparation of thyroxin is neutralized, the impurities appear to influence thyroxin in a manner similar to the addition of the acetyl group to the molecule, and at the proper hydrogen ion concentration, which is very nearly the neutral point, there is a reaction between the impurities and thyroxin resulting in a breaking off of iodine. Temperature greatly influences this reaction. If carried out in the cold solution, no destruction of thyroxin occurs.

Another effect of temperature during acidification is probably that of polymerization. Indole compounds polymerize very readily in the presence of acid. Thyroxin in pure state is slowly changed by boiling in strong acid, but when only partially purified the action of strong acid and heat brings about such a deep seated alteration in the molecule that its physiologic activity is destroyed.

2. Effect of Heating the Alkaline Hydrolysis Solution in the Presence of Metal.—Thyroxin is very susceptible to reduction. An alkaline solution of thyroxin may be completely decomposed by heating with metallic zinc; the iodine is broken off from the molecule. Without doubt, the other metals, iron, copper, tin, lead, German silver, and, in fact, all metals except nickel, gold, silver, and platinum, react in the same manner, causing the destruction of thyroxin by reduction.

3. Effect of Carbon Dioxide.—There are two distinct actions produced by carbon dioxide. One is partial purification of thyroxin by precipitation from an alkaline solution. If an alkaline solution of partially purified thyroxin is treated with carbon dioxide until no further precipitation is produced, and the precipitate is removed by filtration, a large amount of material may be precipitated in the filtrate by the addition of a stronger acid. Thyroxin is precipitated by carbon dioxide more completely than, and at a point far in advance of, certain of the impurities. The effect of carbon dioxide as a precipitant depends on the ratio between the amount of thyroxin and the amount of impurities present. In the first solution of the acid-insoluble constituents

of the hydrolyzed thyroid proteins, carbon dioxide will not precipitate any thyroxin. As the impurities are removed, the precipitation of thyroxin by carbon dioxide becomes more and more complete until in a solution of pure thyroxin the precipitation with carbon dioxide is quantitative. This influence of the impurities on the precipitation of thyroxin by carbon dioxide is one of the most striking examples of the effect of impurities on the chemical properties of thyroxin.

Another influence of carbon dioxide on the separation of thyroxin is more deep seated and was, at first, very difficult to explain. Thyroxin is soluble in sodium hydroxide with the formation of the disodium salt. If carbon dioxide is passed into such a solution, the hydroxy group gives up its sodium and exists in the free form. The carboxyl group, however, retains the sodium in the form of a mono-metal derivative. This is far less soluble than the di-metal derivative and readily crystallizes from the solution. When partially purified thyroxin, which exists in the open-ring form, is treated in this manner with sodium hydroxide and carbon dioxide, the optimum conditions are produced for the closing of the ring, and after standing for a considerable length of time the ring closes with the crystallization of the mono-metal salt of the closed-ring form of thyroxin.

4. Effect of Temperature during the Treatment of a Solution with Carbon Dioxide.—This is closely related to, and is probably identical with, the effect of temperature on acidification of a solution with a mineral acid. The effect is the breaking off of iodine from the thyroxin molecule, and the mechanism is undoubtedly a reduction due to the presence of impurities and accelerated by the increase in temperature, the carbon dioxide functioning merely in producing the proper hydrogen ion concentration for this reaction to take place.

5. Effect of Different Samples of Desiccated Thyroid.—One of the most important reasons for the failure to separate thyroxin consistently was the variability of the samples of desiccated thyroid used. Analysis of the samples obtained at different times of the year shows that in the winter months, January, February, and March, the iodine content of the glands may be so low as to make the isolation impracticable. During the summer months the thyroxin content of the gland increases from 400 to

500 per cent and allows a much more liberal and much simpler purification of thyroxin. The amount of impurities present appears to be more nearly constant, so that in the winter months the problem is greatly complicated by having to deal with approximately the same amount of impurities and with a greatly diminished amount of thyroxin. It is significant that the real progress was made with the problem only when a satisfactory sample of desiccated thyroid was obtained, but this was demonstrated only after the completion of the work. Another influence, which undoubtedly is very important, not only in the isolation of the substance but in the consideration of the therapeutic value of any sample of desiccated thyroid is the state of preservation of the thyroid proteins. Thyroxin has been shown to contain two carboxyl groups and one amino group, when existing in open-ring form, in which state it undoubtedly does exist in the thyroid proteins. Deamination and decarboxylation by bacteria are well known, and it seems highly probable that some samples of desiccated thyroid are without therapeutic value because of bacterial decomposition. Bacterial action could very readily result in deamination and decarboxylation of thyroxin, which would render the substance without physiologic activity although the iodine content of the material would not be altered.

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STUDIES ON THE BEHAVIOR OF INULIN IN THE ANIMAL BODY.

II. INULIN IN THE ALIMENTARY CANAL.

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During the spring of 1918 the cultivation of the Jerusalem artichoke *Helianthus tuberosus*, as a crop likely to yield large quantities of food in return for a comparatively small investment in land and labor, was advocated in the popular scientific press (1). Later, Howard (2) again directed attention to the possibilities of this plant as an aid in the solution of the food problem in France. He stated that its use as a substitute for the potato had been recommended by prominent French agriculturalists who were advising its introduction into all parts of France.

Since the principal reserve carbohydrate of the artichoke is inulin, which differs from starch in that it is a polymer of levulose, and the inulin is present to the extent of 10 to 12 per cent of the weight of the tubers, the question of its real food value is again raised. Almost 40 years ago inulin was first suggested as a substitute for starch in the diet of the diabetic; the chief reason for its use being the fact that, when fed to a diabetic, this carbohydrate gives rise to no increased reducing sugar in the urine. A certain school of German therapists, notably Strauss (3), has therefore advocated the use of vegetables containing inulin in diabetic dietaries in such a way that Continental text-books on dietetics usually recommend them. Lewis (4) has, however, pointed out that the failure of this carbohydrate to give rise to sugar in the urine of a diabetic may be due to the fact that it is not utilized at all. Thus the evidence as to the value of inulin as a source of energy for the animal body is by no means conclusive.

It is generally conceded that there is no enzyme secreted by the organs of the mammalian digestive tract which is capable of hydrolyzing inulin (5). An experiment of Sandmeyer (6) in which he fed 80 gm. of inulin to a depancreatized dog resulted in the recovery of over 46 gm. in the feces. Lewis (4) found no inulin in the feces after the ingestion of 60 gm. by a healthy man, but he observed a very marked increase in the intestinal

fermentation.[1] Goudberg (7) on the other hand, has presented data showing that inulin causes a slight but prolonged rise in the respiratory quotient in man and indicating that it is in some way utilized as a source of energy. Various investigators, notably Bierry (8), have concluded that, since inulin is readily hydrolyzed by dilute HCl at 37°C., the acid of the gastric juice may convert it to levulose, permitting its absorption and utilization in this form. Goudberg (7) has also pointed out two other possibilities; namely that the inulin may be absorbed as such and burned in the tissues; or that it may be changed to organic acids by the bacteria of the intestinal tract, and burned without passing through the intermediate stage of monosaccharide. The fact that no inulase has ever been found in any of the tissues or in the blood of mammals speaks against the possibility of parenteral utilization (9) as do also the experiments of Mendel and Mitchell (10). Thus it would seem that, in order to be utilized by the animal body, inulin must be either hydrolyzed by the acid of the gastric juice, or in some way decomposed by the bacteria of the intestinal tract.

The investigation to be reported here has dealt with (*a*) the hydrolysis of inulin *in vitro* by concentrations of HCl which might be expected in normal gastric juice, (*b*) the action of gastric juice on inulin *in vivo*, and (*c*) the demonstration of the presence of an inulase in fecal extracts.

Action of HCl on Inulin in Vitro.

Chittenden (11) stated that inulin was hydrolyzed by very weak acid (0.05 to 0.2 per cent HCl at 40°C.) both when this was present as free HCl and when it was combined with protein. The details of his experiments are not given. Bierry (8) and Bieri and Portier (12) found that the various digestive fluids acted upon inulin only when acid was present, and that they obtained hydrolysis with HCl alone at body temperature. But, with the single exception of a few control experiments of Dean (13) which dealt primarily with the optimum conditions for the action of the inulase of *Aspergillus niger*, there seems to be no record in the literature of a careful quantitative study of the hydrolysis effected by acid at body temperature under definite conditions of concentration and time.

[1] In a series of unpublished experiments on rabbits, the writer has also observed that inulin feeding is always followed by very marked gas formation in the intestines.

Such a study was, therefore, attempted with some concentrations of HCl which might be expected to be found in gastric juice under normal conditions. A series of experiments was

TABLE I.

*Hydrolysis of 0.5 Per Cent Inulin Solutions by HCl at 37°C.**

Strength of HCl.	Time in incubator.	Levulose from 10 mg. of inulin (apparent value).	Levulose from 10 mg. of inulin (corrected† for color of unhydrolyzed‡ inulin).
per cent	*hrs.*	*mg.*	*mg.*
0.10	1	1.0	0.2
0.10	3	2.0	1.3
0.10	4	2.5	1.9
0.10	5	3.0	2.4
0.10	6	3.5	3.1
0.10	7	4.0	3.6
0.25	1	1.6	0.9
0.25	2	2.7	2.1
0.25	3	3.5	3.0
0.25	4	4.6	4.1
0.25	5	5.6	5.2
0.50	1	2.3	1.7
0.50	3	5.9	5.5
0.50	4	7.4	7.4
0.50	5	8.3	8.3
0.50	6	8.8	8.8
0.50	7	9.3	9.3
0.50	8	9.7	9.7

* The inulin used contained 10 per cent moisture. Therefore, results are calculated on the basis of 1 mg. moist inulin = 1 mg. levulose since, theoretically, 0.9 mg. inulin = 1 mg. levulose, after hydrolysis.

† The corrections for the color from inulin are less where the degree of hydrolysis is greater, since the depth of color developed by the levulose made it necessary to use smaller samples for analysis.

‡ The amount of unhydrolyzed inulin at any time was determined by a method of approximations, starting with the values taken from results given in the third column above.

carried out, the details of which are indicated in Table I; each experimental tube containing 2 cc. of a 1 per cent inulin solution and 2 cc. of acid of concentration double that to be tested. The tubes were placed in an incubator at 37°C., and duplicate tubes

from each set removed for analysis at stated intervals. The contents of each were at once neutralized exactly to methyl red, made up to definite volume, and aliquots which decreased in

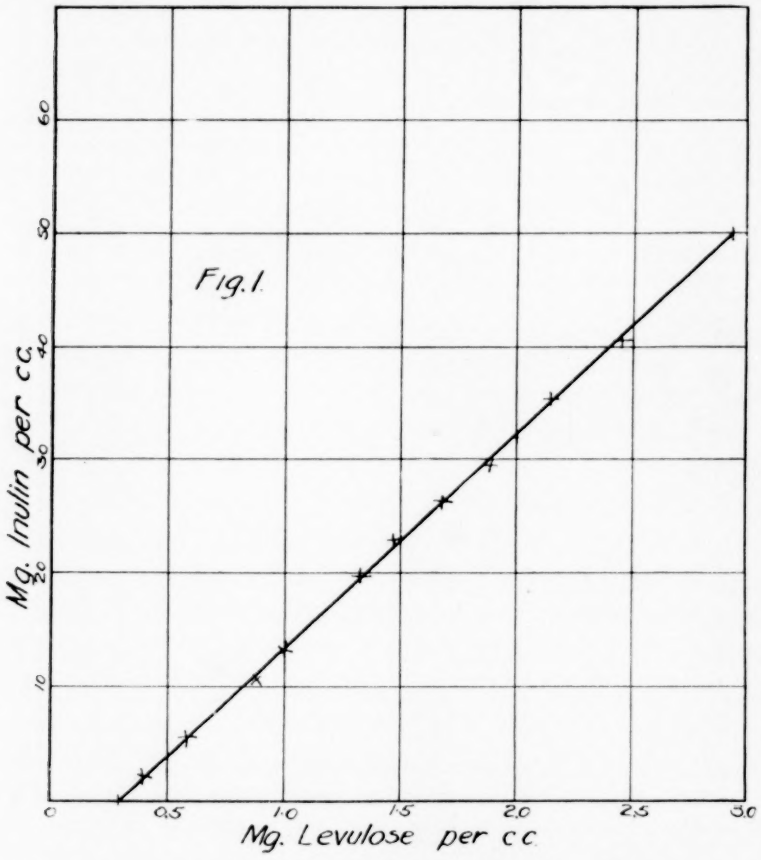

FIG. 1. Correction Curve. Color developed by inulin solutions treated as in Benedict's method for blood sugar. Expressed in terms of the amounts of levulose producing the same color when so treated.

volume with the increase in the degree of hydrolysis taken for analysis. This was made according to a slight modification[2] of

[2] The samples were, in each case, made up to 6 cc. with distilled water diluted to the mark in a 25 cc. volumetric flask with the picrate-picric

the method described by Benedict (14) for the determination of sugar in blood. The results of these analyses are shown in Table I and Figs. 1 and 2.

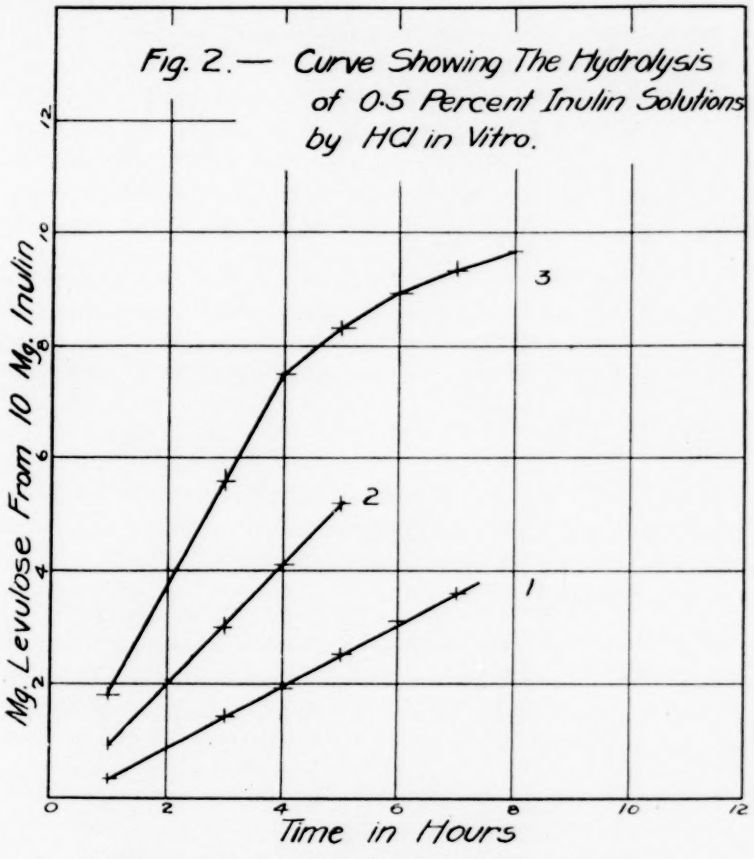

FIG. 2. Curve 1, hydrolysis by 0.10 per cent HCl.
" 2 " " 0.25 " " HCl.
" 3 " " 0.50 " " HCl.

acid solution, mixed, 8 cc. samples removed and heated, after the addition of 1 cc. each of a 20 per cent solution of sodium carbonate, for 10 minutes in a boiling water bath. They were then cooled, diluted to 25 cc., and the color compared in a Duboscq colorimeter with a picramic acid standard which had been adjusted to match the color from 4 mg. of pure levulose treated in the same way.

It is necessary to mention, in connection with the interpretation of these results, that, while levulose may be determined by the Benedict method in the presence of small amounts of inulin, if the concentration of this substance exceeds 3 mg. per mg. of levulose (15) a correction must be made for the color of the inulin. For the sample of inulin used in this and later experiments, this correction curve has been determined experimentally, and is shown in Fig. 1. The correction is practically negligible where the degree of hydrolysis has been great enough to make considerable dilution necessary to secure a sample within range of the color standard; but where the amount of unhydrolyzed inulin present is comparatively large, it may be important.

The results of these experiments indicate that there is appreciable hydrolysis of inulin at 37°C. by concentrations of free HCl known to be present in normal gastric digestion, but that in the 2 or 3 hours which inulin may reasonably be expected to remain in the stomach the degree of this hydrolysis is comparatively small. Moreover, the dilutions used were such that these experiments probably furnished more favorable conditions for the hydrolysis than might be expected in an actual gastric digestion.

Action of the Gastric Juice on Inulin in Vivo.

It was considered that results which would be more typical of conditions during digestion might be obtained from the study of the behavior of inulin introduced into the stomach. Very few such experiments have been reported in the literature. Külz (16) fed 40 per cent inulin solutions after gastric lavage. On removal of the stomach contents, 20 minutes later, he observed no hydrolysis. Bierry (8) and Goudberg (7), on the basis of evidence furnished by incubation of inulin with gastric juice *in vitro*, believe that the amount hydrolyzed by the gastric juice is considerable.

The general plan of the present series of experiments was as follows: The subjects were fed inulin meals, and samples of the stomach contents removed from time to time through a Rehfuss stomach tube. On each of these samples, the "free" acidity was determined by titration against standard NaOH, using dimethylaminoazobenzene as an indicator; the "total" acidity was deter-

mined in like manner, using phenolphthalein as an indicator; and the hydrogen ion concentration was estimated roughly by the indicator method of Clark and Lubs (17). The amount of levulose resulting from the hydrolysis of inulin in the stomach was determined by a modified Benedict method; measured portions of the samples being neutralized immediately after removing from the stomach, made up to 6 cc. with distilled water, the protein precipitated with sufficient picrate-picric acid solution to make a total volume of 25 cc., and the sugar in the filtrate determined by the procedure described above. The total amount of carbohydrate present in each sample was determined in like manner, after hydrolysis of the inulin with 0.5 per cent HCl at 100°C.; while a portion of each sample was incubated at 37°C. for an additional 2 hours, and the levulose determined as before. The results represent, therefore, an estimate of the amount of inulin remaining in the stomach at definite intervals after ingestion, the percentage of this which was hydrolyzed, and the amount which was capable of being hydrolyzed by the acid present if this were given an additional 2 hours to act upon the inulin.

In the first few experiments, the inulin was given in solution as a "water meal," after removing the residuum and washing out the stomach with distilled water. It was soon evident that, when given in this way, the inulin did not stay in the stomach long enough to approximate normal conditions. In order to secure longer retention of the gastric contents, and, if possible, to stimulate the production of a greater degree of acidity, a meal consisting of the whites of several eggs was given to each of the subjects of subsequent experiments before the tube was swallowed. Since it was desirable to subject the inulin to the action of the highest concentration of acid produced, the inulin was not given until 20 minutes after the ingestion of the egg meal. Samples were removed for analysis at 20 minute intervals thereafter.

Eight experiments were carried out altogether, using seven different subjects, all apparently healthy men and women, students between the ages of 18 and 26 years. One of these experiments was, however, not typical; for, although it was continued for 2 hours and 40 minutes, there was no development of "free" and no increase in "combined" acidity; and, as was to be expected, no hydrolysis of the ingested inulin, even after 2 hours in the

incubator, following removal of the samples from the stomach. The stomach was also very slow in emptying itself, since the concentration of inulin remained constant for an hour, and decreased

TABLE II.

Inulin Feeding after a Meal of Egg White: Experiment 3.

Subject: H. F., student, athlete, weight, 72 kg. After a 12 hour fast the whites of three eggs were given; followed, in 15 minutes, by 7.5 gm. of inulin in 225 cc. of water, given through the Rehfuss stomach tube. Samples of the gastric contents were removed at 20 minute intervals thereafter.

Time from ingestion of inulin to removal of sample, *min.*	20	40	60	80
"Free" acidity = cc. 0.1 N NaOH per 100 cc. gastric contents.	9.3	50.0	33.6	24.3
"Total" acidity = cc. 0.1 N NaOH per 100 cc. gastric contents.	23.6	82.0	50.0	37.1
pH* (approximate).	4–6	1–2	1–2	1–2
Total carbohydrate as mg. levulose per cc. gastric contents.	8.23	4.71	1.62	0.74
Mg. levulose per cc. gastric contents at time of removal of sample (corrected for color of unhydrolyzed inulin).	0.26	0.16	0.11	0.07*
Mg. levulose per cc. gastric contents after 2 hrs. additional incubation at 37°C. (corrected for the color of unhydrolyzed inulin).	0.35	0.74	0.18	0.09*
Per cent of total inulin in gastric contents hydrolyzed in stomach.	3.1	3.4	6.7	9.0*
Per cent of total inulin in gastric contents hydrolyzed after incubation for an additional 2 hours.	4.3	15.7	11.1	12.1

* Approximate values only, because of the differences between the depth of color of the standard and the unknown.

very slowly thereafter. This experiment seems to give conclusive evidence that, where there is no "free" acidity, there is little or no hydrolysis of the inulin.

In the two experiments where water alone was given with the inulin, there was no measurable hydrolysis of inulin in the time it remained in the stomach, and there was no appreciable quantity

TABLE III.

Inulin Feeding after a Meal of Egg White: Experiment 4.

Subject: J. J., student, young man, weight 65.5 kg. After a 12 hour fast the whites of four eggs were given; followed, after 15 minutes, by 7 gm. of inulin in 200 cc. of water, given through the Rehfuss stomach tube. Samples of the gastric contents were removed at 20 minute intervals thereafter.

Time from ingestion of inulin to removal of sample, *min.*	20	40	60	80
"Free" acidity = cc. 0.1 N NaOH per 100 cc. gastric contents.	0.0	4.3	28.6	38.6
"Total" acidity = cc. 0.1 N NaOH per 100 cc. gastric contents.	8.6	20.0	56.4	72.8
pH* (approximate).	4–5	4–5	3	1–2
Total carbohydrate as mg. levulose per cc. gastric contents.	17.4	12.6	5.5	1.1
Mg. levulose per cc. gastric contents at time of removal of sample (corrected for color of unhydrolyzed inulin).	0.4	0.32	0.17	0.11
Mg. levulose per cc. gastric contents after 2 hrs. additional incubation at 37°C. (corrected for color of unhydrolyzed inulin).	0.66	0.70	0.84	0.83
Per cent of total inulin in gastric contents hydrolyzed in stomach.	1.1	2.5	3.0	9.5
Per cent of total inulin in gastric contents hydrolyzed after incubation for an additional 2 hours.	3.8	5.5	15.2	72.1

*Approximate values only, because of the differences between the depth of color of the standard and the unknown.

of inulin left in the stomach at the end of 1 hour. That the acid present was capable of hydrolyzing the inulin to a considerable degree was shown clearly in one case, where an unavoidable interruption led to the incubation of a set of samples for 32 instead

of the usual 2 hours. With "free" acidity values of 4.5, 23.0, and 24.0 cc. respectively of 0.1 N NaOH per 100 cc. of gastric contents, hydrolysis amounting to 65.5, 66.0, and 71.8 per cent of the inulin in the different samples was obtained.

TABLE IV.

Inulin Feeding after a Meal of Egg White: Experiment 5.

Subject: H. M., student, young woman, weight 79 kg. After a 12 hour fast the whites of three eggs were given; followed, after 20 minutes by 5 gm. of inulin in 150 cc. of water, given through the Rehfuss stomach tube. Samples of the gastric contents were removed at the stated intervals thereafter.

Time from ingestion of inulin to removal of sample, *min.*	15	40	60	80
"Free" acidity = cc. 0.1 N NaOH per 100 cc. gastric contents.	5.0	23.6	13.2	6.6
"Total" acidity = cc. 0.1 N NaOH per 100 cc. gastric contents.	15.5	47.2	32.2	24.8
pH* (approximate).	3–4	3	3	3
Total carbohydrate as mg. levulose per cc. gastric contents.	21.42	9.67	3.90	0.98
Mg. levulose per cc. gastric contents at time of removal of sample (corrected for the color of unhydrolyzed inulin).	0.10	0.35	0.10	†
Mg. levulose per cc. gastric contents after 2 hrs. additional incubation at 37°C. (corrected for the color of unhydrolyzed inulin).	0.10	0.86	0.36	†
Per cent of total inulin in gastric contents hydrolyzed in stomach.	0.5	3.6	2.5	†
Per cent of total inulin in gastric contents hydrolyzed after incubation for an additional 2 hrs.	0.5	8.8	9.2	†

* Approximate values only, because of the differences between the depth of color of the standard and the unknown.

† Out of range of color standards.

The results of the six experiments in which inulin was given after a meal of egg white indicate that there is some hydrolysis

of inulin in the stomach, especially if there is considerable "free" acidity, and the stomach empties itself slowly. This can, however, be expected to represent only a comparatively small fraction—seldom more than 10 per cent, and usually much less—of the ingested inulin. There is, however, the possibility that levulose leaves the stomach faster than unhydrolyzed inulin, and that the degree of hydrolysis is greater than it appears. In order to condense the data, details of three representative experiments only are given in the tables.

It is evident, therefore, that the greater part of the inulin leaves the stomach as such, and that, if this carbohydrate is digested at all, a large part of this digestion must take place elsewhere.

Inulase in Fecal Extracts.

Inulin, on leaving the stomach, is subjected to the action of the pancreatic juice, bile, and intestinal juice. The work of Bierry (8) seemed to have established clearly the fact that none of these could hydrolyze this carbohydrate. It remained, therefore, to investigate the action of the microflora of the lower part of the intestine. The inulases of the molds, *Aspergillus niger* and *Penicillium glaucum*, have been studied carefully by Dean (13), Boselli (18), and Kiesel (19), while Grafe and Vouk (20) have studied the possibilities of various types of yeasts as inulase formers. On the other hand, the literature on the subject of the chemical transformation of inulin by bacteria is fragmentary. It is generally supposed, and there is some experimental evidence (21) in support of the view that inulin is broken down by bacteria of the types normally present in the intestine; with the formation of various organic acids, some of which, at least, are utilized by the animal body without the intermediate formation of levulose.

This investigation has dealt only with the demonstration of the presence of an inulase in fecal extracts. Samples of fresh feces were incubated with about ten times their weight of distilled water in the presence of chloroform for 24 hours at 37°C., in order that autolysis might take place and any endoenzymes present be set free. The extracts were then filtered through sterile Berkfeldt filters into sterile flasks, the completeness of the sterilization being checked by plating out 0.5 cc. portions on dextrose agar. 5 cc. sam-

ples of the extracts were then measured into each of a series of test-tubes, and 5 cc. of a 4 per cent inulin solution added, using all precautions to insure aseptic conditions. As an additional

TABLE V.

Effect of Incubating Inulin with Sterile Extracts of Feces.

Source of sample.	Duration of incubation.	Reduction.			
		Benedict's solution.		Fehling's solution.	
		Boiled control.	Unboiled sample.	Boiled control.	Unboiled sample.
	hrs.				
Human feces I. (Normal adult, mixed diet.)	24	−	+++	−	+++
	48	−	+++	−	+++
	72	−	+++	−	+++
Human feces II. (Normal adult, low protein, meat-free diet.)	24	−	++	−	++
	48	−	++	−	++
	72	−	++	−	++
Human feces III. (3 yr. old child, milk diet.)	24	−	+	−	+
	48	−	+	−	+
	72	−	+	−	+
Dog feces I. (Dry.)	24	++	++	++	++
	48	++	++	++	++
	72	++	++	++	++
Dog feces II. (Moist.)	24	−	−	−	−
	48	−	−	−	−
	72	−	−	−	−
Guinea pig feces I.	24	−	−	−	−
	48	−	−	−	−
	72	−	−	−	−
Guinea pig feces II.	24	−	−	−	−
	48	−	−	−	−
	72	−	−	−	−

protection from outside infection, the contents of each tube were covered with toluene. Half the tubes of each series were then heated to the boiling point to kill any enzymes present, and the

entire series incubated at 37°C. At the end of 24, 48, and 72 hour periods, tubes were removed from the incubator, and tests for reducing sugar made with the ordinary Fehling's and Benedict's qualitative reagents. Results are shown in Table V. In two experiments where the inulin was found to be hydrolyzed, quantitative determinations of the total carbohydrate present in the control tube and the tube containing the unheated extract were made by the modified Benedict picric acid method described before. The amount of carbohydrate in the two tubes was found to be the same; hence there was no decomposition of inulin beyond the stage of reducing sugar.

These results indicate that some microorganism which elaborates an enzyme having the power to hydrolyze inulin to the stage of reducing sugar may be present in normal human feces. Whether this is a stage in the breakdown of inulin by one type of bacteria which has the power to carry the decomposition further, or whether the inulin is hydrolyzed to levulose by one type of organism, and this sugar fermented by another, cannot be determined without further study. It would be very interesting to find out whether appreciable quantities of levulose formed in this way are ever absorbed from the intestine, or whether the further decomposition takes place so rapidly that this is impossible. The marked gas formation which always follows inulin ingestion may be taken as evidence in favor of the latter view.

SUMMARY.

Inulin may be hydrolyzed *in vitro* by concentrations of HCl present normally in gastric juice, if the hydrolysis is continued for a sufficiently long time. The amount of hydrolysis in 2 or 3 hours, the period which it might reasonably be expected to remain in the stomach, is comparatively small.

In a series of eight experiments on the gastric digestion of inulin *in vivo*, where the inulin was given with a water meal or after a meal of egg white, the maximum hydrolysis obtained after 1 hour and 40 minutes in the stomach was 9 per cent of the total carbohydrate present. The samples of gastric contents removed at this time, however, never contained more than 3 per cent of the inulin present at the end of 20 minutes after ingestion. An additional 2 hours incubation at 37°C. *in vitro* gave a maximum

hydrolysis of 65 per cent and an average hydrolysis of 49 per cent of the inulin remaining in the stomach at the time the samples were taken. This indicates that, while the acidity of the gastric juice is usually sufficient to make a fair degree of hydrolysis possible, inulin is seldom retained in the stomach long enough to be changed to levulose by the HCl of the gastric juice. Where there is no "free" acidity, there is little or no hydrolysis.

The presence of an enzyme capable of producing a reducing sugar from inulin has been demonstrated in sterile extracts of three samples of human feces from radically different types of diets. Negative results have been obtained with samples from a dog and from guinea pigs.

The writer wishes to acknowledge her indebtedness to Professor H. B. Lewis of this laboratory for his assistance in the gastric digestion experiments, and for many suggestions and criticisms.

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HEMATO-RESPIRATORY FUNCTIONS.*

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I. The CO_2 Diagram of the Blood.

The balance of acid and alkaline elements in the blood is not fixed, but fluctuates as the blood passes through areas of varying tension of CO_2. A determination of the hydrogen ion concentration, C_H, or the alkali bicarbonate establishes merely one of the values which the blood may have. It fixes only a single point in an otherwise unknown or only vaguely apprehended line.

This line, as we shall show, is the CO_2 dissociation curve of the blood: a curve expressing all the values of which the particular blood under examination is capable. (For literature see Christiansen, Douglas, and Haldane (1).) Upon this curve it is usually easy to fix the arterial and venous values: the limits between which not only the CO_2 tension and content, but also the C_H and

* The greater part of the work both for this and for succeeding papers was done under the War Gas Investigations and the Aviation Investigations of the Bureau of Mines, and later under the Chemical Warfare Service, U. S. Army, by the physiological section, including besides the authors, R. W. Brooks, S. R. Detwiler, G. C. Ellerbeck, H. Kahle, and H. B. Robb.

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alkali in use, oscillate each time that the blood makes the round of the circulation.

The curve, *A–V* in Fig. 1, is plotted from analyses of the CO_2 which the blood contains after being equilibrated at 38°C. with various mixtures of air and CO_2. The tensions of CO_2 in these atmospheres are controlled by analysis and are expressed by the abscissæ. The corresponding ordinates show the volumes per cent of CO_2 which the blood contains at these tensions.

We use for the equilibrations CO_2 tensions of 40, 72, and 18 mm., always in this order; for we find that a slight hemolysis is often induced by exposing the blood to the low tension 18 mm. The liberated hemoglobin then acts like a strong acid. It may lower by several volumes per cent the CO_2 capacity of the blood when it is later equilibrated to tensions of 40 and 72 mm.

Defibrination sometimes has a similar effect.

In all our observations a volume of 10 cc. of blood was drawn under liquid petrolatum of high viscosity in a vessel containing 0.05 gm. of powdered ammonium oxalate of tested purity.

Control experiments have shown that withdrawals of blood in considerably greater amount than in any experiments here reported have no appreciable effect on the level or form of the dissociation curve.

In another set of controls we injected fluid, saline or water, in volumes equal to the acid employed in the experiments here reported. There was no effect upon the level or form of the dissociation curve.

Negative results occurred also in control experiments with slight loss of blood and replacement by saline.

No general anesthetic was used in any of our experiments, as the respiratory disturbances would have invalidated the data. Morphine was given in only one experiment (No. 17). The blood vessels were exposed under local anesthesia,—usually cocaine. The character of the data here published affords absolute proof that the animals neither suffered any pain or discomfort, nor were they at all anxious or irritated mentally; for these conditions would have affected the breathing and would show in the CO_2 tensions.

Once the dissociation curve of a blood has been determined, one coordinate is of course sufficient to fix a point. For example, the CO_2 tension of the alveolar air, the pulmonary arterial air (2), when applied to the curve, gives the quantity of CO_2 in volumes per cent in the arterial blood; while an analysis of the quantity of CO_2 in a sample of venous blood enables us to draw from the curve the exact tension of CO_2 in the tissue capillaries through which the blood last passed.

When the arterial and venous points, *A* and *V* in Fig. 1, determined from the arterial and venous CO_2 contents, have been placed on the curve, they show both the quantity of CO_2 (in cc.) which each unit volume of blood transports from the tissues to the lungs, and the extent to which the tension of CO_2 in the tissue capillaries must rise above that in the lungs, in order that the blood may take up this amount of the gas and carry it away.

It will become evident also, as we proceed with the study of the properties and relations of this curve, that when it lies at a low level the breathing of the man or animal, even while at rest, must necessarily be abnormally great. It will appear also that a flat curve, one with little rise of CO_2 capacity, must cause an abnormally high CO_2 tension in the tissues; *i.e.*, a venous and tissue acidosis. A steeply ascending curve, on the contrary, may afford one of the conditions of health and capacity for great exertion, since the volume of blood which the heart would need to pump in order to transport a certain amount of CO_2 would be much less than when the curve is flat.

These bearings of the curve, important as they are, are not however just now its most significant aspect. Present interest in the study of the blood centers particularly about the balance of acids and alkalies; *i.e.*, the condition which in a less complicated and more easily analyzable fluid we should estimate directly as C_H, the concentration of hydrogen ions. Except for special purposes however the methods for direct determination of the C_H of the blood are as yet somewhat esoteric. It is fortunate therefore that, as Hasselbalch (3) has shown, practically equivalent,—in some respects more than equivalent,—information may be obtained from analyses of the amount of CO_2 which, under accurately defined conditions of temperature and tension, the blood will contain.

For such analyses several simple, rapid, and sufficiently accurate methods are now available. That of Van Slyke (4) has come into common use and is excellent for plasma. For whole blood the method described by Henderson and Morriss (5) is the easiest and quickest. As the description of this method given by them was only of a preliminary character one of us will shortly publish in this journal an elaboration and critique of it based on some 20,000 analyses.

FIG. 1. The CO_2 diagram of normal blood at 38°C., from the data of Experiment 1.

The line *OS* shows the CO_2 dissolved at all tensions. It is positive, not negative.

The CO_2 dissociation curve *A–V* is plotted from the analyses of the blood, equilibrated to CO_2 tensions of 40, 72, and 18 mm. (in this order) at body temperature. For the total CO_2 content, *i.e.* combined plus dissolved, the line *OS*,—not *OB*, is used as the base. The elevation of the curve above *OB* shows the alkali, measured in volumes per cent CO_2 combined, called into use in the blood by various tensions of CO_2.

Using the CO_2 content of the arterial and that of the venous blood respectively as ordinates and the line *OS* as a base, the points *A* and *V* on the dissociation curve are found. The abscissæ of these points are the corresponding tensions of CO_2,—either arterial, that is pulmonary alveolar, or venous. These tensions divided by 760 and then multiplied by the solubility coefficient of CO_2 for whole blood at 38°C., 0.511 (Bohr), give the CO_2 dissolved. Thus

(Tension CO_2 in mm. $\div$ 760) $\times$ 0.511 = volume of CO_2 dissolved (in per cent).

CO_2 content $-$ CO_2 dissolved = CO_2 combined.

CO_2 dissolved $\div$ CO_2 combined = *R* (in per cent).

$R \times$ Char. = C_H7.

The dissociation characteristic (Char.) of arterial blood is approximately 8 (see Table I); even when the blood is completely deoxygenated it is probably never less than 7 (cf. Parson's observations in Table I). Two scales for $C_H \times 10^{-7}$, or as we write it C_H7, have been placed around the upper right hand corner of the diagram, and show the range of variation between *A* and *V* through which the blood oscillates in the circulation. The ends of the scales 0.2 and 1.4 are probably beyond vital limits.

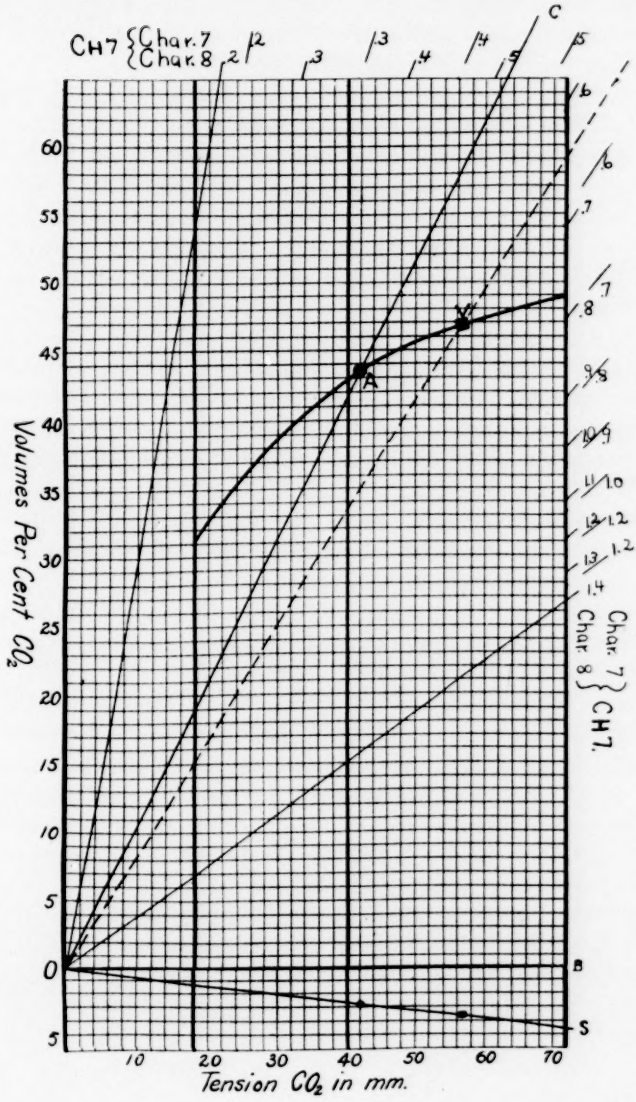
CH7 {Char. 7 / Char. 8
C
A
B
S
Volumes Per Cent CO_2
Tension CO_2 in mm.
Char. 7 / Char 8 } CH7.

FIG. 1.

Walter (6), working in Schmiedeberg's laboratory 42 years ago, measured the alkalinity of the blood by the amount of carbon dioxide which it would hold.

For the purpose of estimating in this way the degree of "alkalinity" all the CO_2 of the blood is assumed to be distributed between two general forms,—that in simple solution and that combined with alkali as bicarbonate, chiefly $NaHCO_3$. No acid stronger than carbonic can exist in significant amounts free in the blood; for all stronger acids, even when directly injected, are neutralized by the sodium bicarbonate present in the plasma with liberation of CO_2.

When a sample of blood has been equilibrated to a certain tension of CO_2 the amount of the gas dissolved (as $CO_2 + H_2CO_3$) is supposed to obey the law of Henry with a coefficient of solubility a little less than that of water. The ratio of H_2CO_3 to dissolved CO_2 is assumed to be constant. (Bohr (7) calculated the solubility of CO_2 in whole blood at 38°C. as 0.511, in plasma 0.541, and in corpuscles 0.450. From the first figure we derive the factor which we commonly use 0.0672 volumes per cent of CO_2 dissolved for each mm. of CO_2 tension.)

By subtracting the dissolved CO_2 from the total CO_2 found in the blood by analysis the amount of bicarbonate (chiefly $NaHCO_3$) or combined CO_2 expressed in volumes per cent of the gas is obtained. This subtraction is effected automatically in our diagram by the fact that the line *OS*, expressing the dissolved CO_2, although drawn below the base line, indicates positive values. The total CO_2 found in the blood by analysis is measured upward from *OS* and marked as a point in the dissociation curve. The combined CO_2 (chiefly $NaHCO_3$) is therefore shown by the height of this point above the base line *OB*.

When the CO_2 dissociation curve is plotted with this subtraction it becomes an expression of what may be conveniently termed the alkali in use. We use this as a more general expression than "alkaline reserve" (8), for it applies to the blood for all tensions of CO_2, and avoids the erroneous implication that the sodium bicarbonate in the blood is a fixed and unchanging quantity. On the contrary, as Zuntz (9) was the first to recognize, it increases as the blood takes up CO_2 in its passage through the active tissues. It decreases again as the blood loses CO_2 in the

lungs. As Hamburger (10) in particular has shown, the corpuscles, and to a less extent the proteins of the plasma, are able under an increased tension of CO_2 to absorb and, so to speak, to hide chlorine. Sodium is thus freed from NaCl to form $NaHCO_3$, while chlorine (in ionic form) passes into the corpuscles; and this chlorine is paid back to the plasma to form NaCl again as the blood passes through the lungs, for otherwise the diffusion of CO_2 into the air would leave the blood too alkaline.

Thinking of the CO_2 dissociation curve as an expression of the varying amount of alkali in use, we see then that it bears a definite relation to the C_H of the blood. In fact it is the complete expression of the conditions which control and determine the C_H. By the application of the principles of physical chemistry, L. J. Henderson (11) has shown theoretically that the C_H of the blood should vary in direct proportion with the ratio dissolved CO_2: combined CO_2. Thus if K is a constant the acid-base equilibrium is expressed by his equation:

$$C_H = K \frac{H_2CO_3}{NaHCO_3}$$

This theory has been confirmed experimentally by the measurements of Hasselbalch and his coworkers (see Table I), by Lundsgaard, and by others (for literature see (12)) who have performed the difficult and extremely valuable service of determining the C_H and the CO_2 content of blood equilibrated to measured tensions of CO_2.

Significance of the OC Line.

In studying some problems of asphyxia and acidosis, we had occasion to plot a series of CO_2 dissociation curves. The data were from the blood of a dog which had received a considerable amount of HCl by slow intravenous injection. With increasing neutralization of the $NaHCO_3$ of the blood the curves fell to progressively lower levels. At the same time the points in the curves corresponding to the arterial blood (the dots on Curves 1, 2, 3, and 4 in Fig. 2) moved further and further to the left. Furthermore, when these arterial points were connected, all were found to lie on, or close to, a diagonal straight line, and

FIG. 2. The CO_2 diagram of the blood during and after administration of HCl. From the data of Experiment 2.

The dots on *OS* and on the dissociation curves indicate the arterial values,—tension and content, dissolved and combined CO_2, and the alkali in use. They show that the compensation at different levels of alkali is almost perfect, as they all fall on, or close to, the *OC* line. A uniform C_H is thus maintained. This is accomplished by the increased respiration, for the dots on the curve Resp. correspond to the arterial points vertically above them. They are evaluated by means of the scale at the lower right side of the diagram. It indicates for all arterial tensions of CO_2 the volume of air breathed per unit mass CO_2 eliminated in per cent of that at 40 mm. tension.

The curve and the positions of the dots indicating the relative volumes of breathing at different times are calculated from the equation:

$$\text{Resp. (in per cent)} = \frac{40 \text{ mm.}}{\text{arterial } CO_2 \text{ tension in mm.}}$$

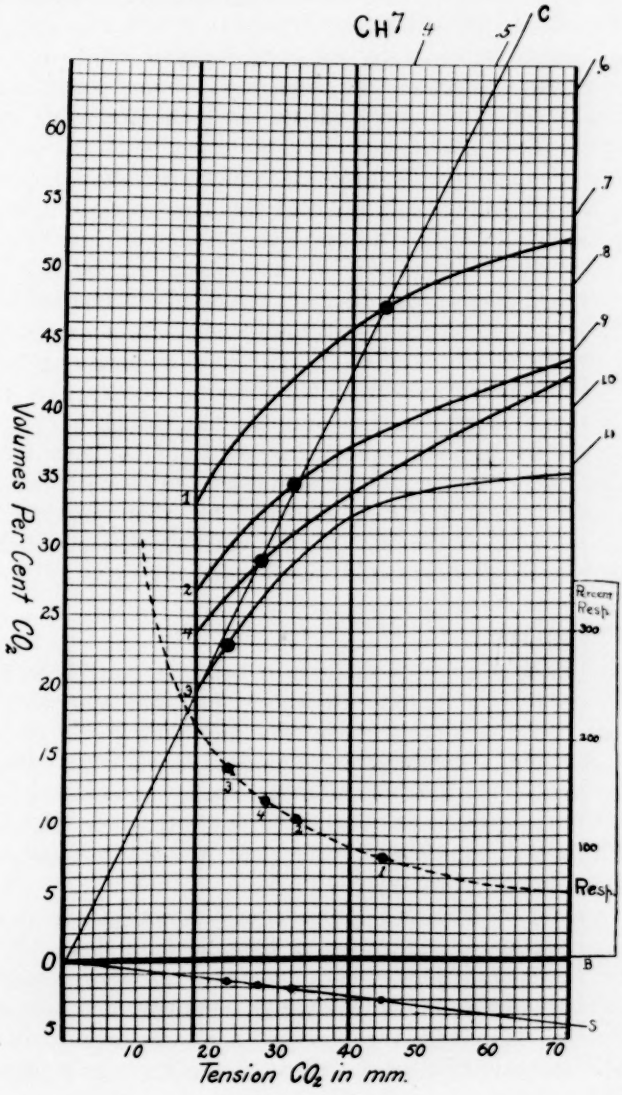
CH7
C
.4
.5
.6
.7
.8
.9
.10
.11
1
2
3
4
5
Room Resp.
300
200
100
Resp.
B
S
Volumes Per Cent CO_2
Tension CO_2 in mm.
0
5
10
15
20
25
30
35
40
45
50
55
60
10
20
30
40
50
60
70

FIG. 2.

this line when continued passed through the intersection of the ordinate and abscissa (the point *O*).

This observation naturally led us to consider what such a line in our diagram expresses. We reached the conception of the CO_2 dissociation curve set forth above, and the following simple explanation of the line.

Such a line expresses the same C_H at every point throughout its length, for all points in it indicate the same ratio of dissolved CO_2 to combined CO_2. We term it the *OC* line or line of *C* value. It expresses the inherent sensitiveness of the respiratory center to its chemical control. It affords a beautiful demonstration that the C_H, and not the total CO_2 in the blood nor the concentration of HCO_3 ions, is normally the hormone of respiration; for evidently as the point indicating the arterial blood moves to the right in this diagram, either along the dissociation curve or upward on the *OC* line, the indicated amount of CO_2, both dissolved and combined, increases. Presumably the concentration of HCO_3 ions increases correspondingly, for this quantity must vary as the sum of the H_2CO_3 and $NaHCO_3$ dissociated in the blood. But respiration varies in the opposite sense.

II. Laws of Respiration.

On the basis of the CO_2 diagram it becomes possible to formulate the laws of the hemato-respiratory relations somewhat more precisely than has hitherto been experimentally justified.

The first law, a modification of that of Haldane and Priestley (13), is: At any one level of the CO_2 dissociation curve or arterial alkali, the pulmonary ventilation varies directly as the mass of CO_2 eliminated, and the alveolar and arterial tension of CO_2 therefore remains constant.

The second law follows from the considerations set forth in the preceding section and is proved in those which follow. It is: At different levels of the dissociation curve the pulmonary ventilation, per unit mass CO_2 eliminated, varies inversely, and the arterial CO_2 tension, therefore, varies directly, as the amount of alkali which in the condition of the blood at the time, as expressed in its CO_2 dissociation curve, will afford the normal C_H.

The third and fourth laws will be considered in detail in a later section of this series. The third is: In conditions of equilibrium

the amount of alkali in use in the arterial blood varies proportionally to the arterial tension of oxygen to which the subject is acclimatized.

The fourth law may be stated provisionally as follows: A low oxygen: alkali ratio tends to stimulate respiration and thus to induce temporarily a low C_H, and a high ratio tends to depress respiration and to induce a high C_H.

The fifth law, demonstrated by our previous work (14) is: The maintenance by respiration of a CO_2 tension affording temporarily a C_H below normal tends to drive alkali out of the blood, and a C_H above normal tends to draw alkali into the blood.

It is the second law which particularly concerns us here. It may be given an approximate mathematical expression. If we use $T_{40}CO_2$ for the tension of CO_2 in mm. in the arterial blood and alveolar air, $Q_{40}CO_2$ for the amount of alkali in use in the arterial blood expressed as volumes per cent of combined CO_2, and 100 Resp. for the volume of the pulmonary ventilation per 5.5 cc. of CO_2 eliminated, the relations by which an unvarying C_H is maintained are

$$T_{40}CO_2 = Q_{40}CO_2 = \frac{1}{100\ \text{Resp.}}$$

The particular conditions here contemplated are such as may occur in a normal subject acclimatized to a barometric pressure of 760 mm. In an individual who at sea level has an alveolar CO_2 above 40 mm. the arterial alkali must be proportionally more than the equivalent of 40 volumes per cent combined CO_2, and the volume of (true) breathing must be correspondingly less than 100 cc. of air per 5.5 cc. of CO_2 eliminated. (The figures 40 and 40 may be slightly too high for T_{CO_2}, and too low for Q_{CO_2}. They are used here for simplicity of exposition.)

It is known that in persons acclimatized to altitude respiration is increased and the alveolar CO_2 is decreased, but the C_H of the blood when equilibrated with the subject's own alveolar air is probably the same or nearly the same as at sea level—as indicated by the mesectic position of the oxyhemoglobin dissociation curve (15, 16). From these facts the inference is justified that a decrease of blood alkali also occurs in general accord with the formula here given.

In so called "compensated acidosis" in nephritics (at sea level) both the alveolar CO_2 and the blood alkali are decreased, and the volume of breathing is abnormally great. From the CO_2 diagram it is evident for example that in compensation an alveolar CO_2 of 30 mm. would correspond to an arterial alkali of approximately 30 and a volume of respiration of 133 per cent.

The interaction of renal with respiratory function in determining the level of the CO_2 dissociation curve, or blood alkali, will afford an interesting problem.

Details of Figures and Protocols.

The proof of the second law is afforded by noting in Figs. 2, 3, and 4 the relations of the *OC* line, the dissociation Curves 1, 2, 3, and 4, the dots upon them indicating the arterial CO_2 values, the curve Resp. and the dots upon it indicating the volume of breathing. The curve Resp. is based on the fact that the alveolar and arterial CO_2 tensions are always identical. It is constructed on the principle, common to all dilutions, that this tension and the volume of the pulmonary ventilation, per unit mass of CO_2 eliminated, vary inversely. The dots are placed on this curve by means of their abscissæ which are the same as those of the arterial points on the corresponding dissociation curves. The ordinates of the dots on Resp. express the respiratory activity. Evidently the volume of breathing requisite to compensate any given amount of blood alkali is indicated by the ordinate of that point on the curve Resp. which has the same abscissa, that is the same CO_2 tension, as the intersection of the dissociation curve with the *OC* line. In fact as examination of Figs. 2, 3, and 4 shows the volume of air breathed is regulated so as to keep the ratio *A* of dissolved and combined CO_2 in the arterial blood on or near the *OC* line, that is at the value *C*, or to restore this value if it deviates. Thus the normal C_H is maintained.

It is to be seen in Experiments 2 to 8 that when acid is injected intravenously the dissociation curve is lowered. If the injection is not too large or too rapid a process of compensation occurs. This consists in an increase of the volume of the pulmonary ventilation to such an extent that the tension of CO_2 and the amount of CO_2 dissolved, and thus the H_2CO_3 in the

blood, are diminished in proportion to the decrease of alkali in use. In this way the arterial CO_2 values are brought to correspond with the values at the intersection of the dissociation curves and the *OC* line. *A* equals *C*, and C_H is thus kept, or restored to, normal.

When, as in Experiments 8 and 9, alkali was injected the dissociation curve was raised, but compensation was effected through a decrease of the pulmonary ventilation which allows the H_2CO_3 in the blood to rise until its ratio to $NaHCO_3$ is again normal and a normal C_H is thus restored.

The details of Experiments 2 to 8 contained in the protocols show that in general the rates and amounts of acid administered were 0.2 to 0.5 cc. per minute up to totals of 35 to 50 cc. in dogs of 11 to 18 kilos. Coincidently with the lowering of the CO_2-combining power of the blood respiration increased sufficiently either to keep the arterial blood at nearly the normal ratio of dissolved CO_2 to combined CO_2 and therefore at the normal C_H, or else quickly to restore normal values when the administration was stopped. In the spontaneous return of the alkali in use toward the normal value the arterial point of the dissociation curve travelled accurately back up the *OC* line down which the administration of acid had previously forced it.

In Experiments 8 and 9 with injections of $NaHCO_3$ the initial depression of respiration was not quite adequate to maintain the arterial point on the elevated dissociation curve at the intersection of the curve and the *OC* line, but when the administration of alkali was stopped respiratory compensation quickly returned, and the arterial point descended the *OC* line as the level of the curve fell again to normal.

Influence of Acids and Alkalies upon Respiration.

It has heretofore been a somewhat puzzling fact that while acids cause a marked increase of respiration alkalies exert a comparatively slight depressing effect. The reason becomes clear from the CO_2 diagram. The steepness of the dissociation curve at the left causes the intersection of the curve with the *OC* line to descend more rapidly than the curve as a whole when acid is injected. The flatness of the curve at the right prevents a corre-

FIG. 3. The CO_2 diagram of the blood, from the data of Experiment 6, showing the effect of a dosage of HCl beyond the system's power of immediate compensation. Thus the arterial point (dot on Curve 2) deviated from the *OC* line and has a C_H7 much above normal, 0.66 instead of 0.497. During recovery the arterial point, dot on Curve 3, returns almost to the *OC* line, $C_H7 = 0.51$.

The dots on the curve Resp. show the volume of pulmonary ventilation corresponding to the arterial points vertically above them. The point *x2'* on the respiration curve shows the (impossible) volume of breathing which would have been necessary in order to bring the arterial values to those at the intersection of the *OC* line and the extrapolated dissociation Curve 2.

The square marks (*V*) on the dissociation curves show the values for the venous blood. Curve 2 shows temporary respiratory decompensation ½ hour after the injection, slowed circulation, increase of arterial C_H, and extremely high venous C_H. Curve 3 shows a marked degree of recovery 3 hours after the injection.

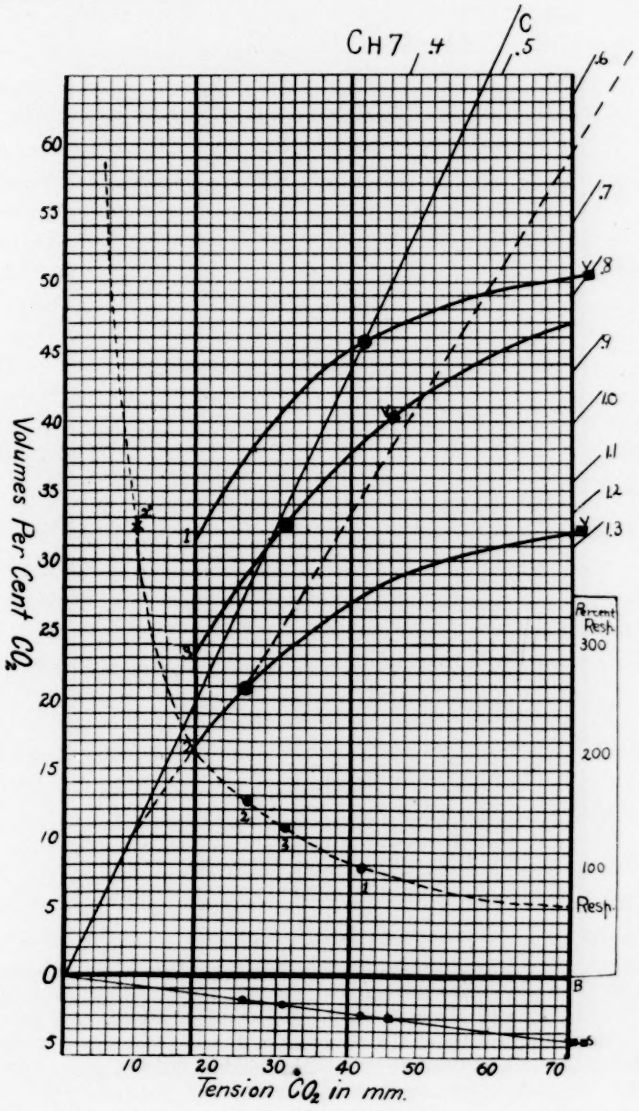
CH7
C
.4
.5
.6
.7
.8
.9
1.0
1.1
1.2
1.3
Percent Resp.
300
200
100
Resp.
B
Volumes Per Cent CO_2
Tension CO_2 in mm.
60
55
50
45
40
35
30
25
20
15
10
5
0
5
10
20
30
40
50
60
70

FIG. 3.

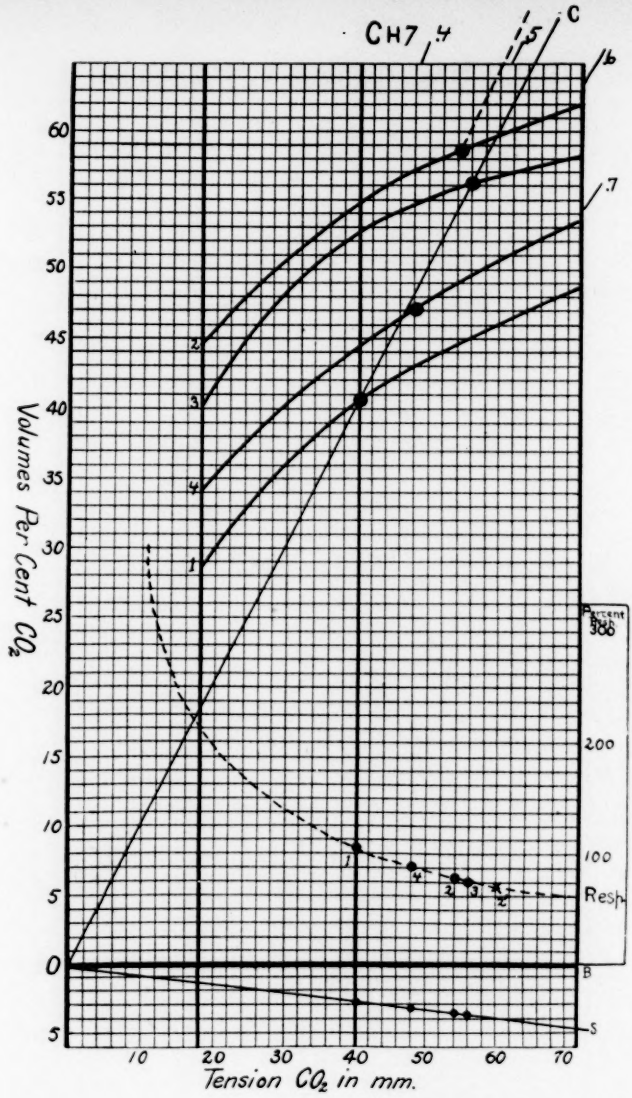

FIG. 4. The CO_2 diagram of the effects of administration of alkali, from the data of Experiment 9. Injection of 96 cc. of 4 per cent $NaHCO_3$ caused elevation of the curve (No. 2) with temporary deviation of the arterial point to the left or alkaline side of the *OC* line. Curves 3 and 4 show how during recovery the arterial point descends the *OC* line.

The dots on the curve Resp. show how slight are the effects of alkali upon breathing in comparison with those of acid in previous experiments, due to the shape of the dissociation curve.

spondingly great change when the curve is elevated with alkalies. Furthermore the curve Resp. verges more and more toward a horizontal line at high tensions of CO_2, and more and more toward a vertical line at low tensions.

. Viewed more closely however the CO_2 diagram shows that in relation to the volume of breathing required on a given dissociation curve to maintain a normal C_H, acids tend to depress respiration and to cause deviation of the arterial values to the right of the *OC* line, while alkalies tend to stimulate breathing and thus to cause deviation to the left. These temporary conditions are in accord with the fourth law.

III. Respiratory Decompensation and Acidosis.

Owing to the convenience of measurements of the alkali of blood or plasma by its CO_2-combining power, it has come about that acidosis and alkalosis are now commonly defined in terms of this factor: the terms low "alkaline reserve" and "acidosis" are used synonymously. The CO_2 diagram indicates however that this habit of non-differentiation may lead to grave error.

Instead of only two forms of deviation from normality, acidosis and alkalosis, the CO_2 diagram shows us that there are theoretically four possible abnormal conditions with all gradations between them: (1) high $NaHCO_3$ and $A > C$, high C_H; (2) low $NaHCO_3$ and $A > C$, high C_H; (3) high $NaHCO_3$ and $A < C$, low C_H; and (4) low $NaHCO_3$ and $A < C$, low C_H. (A is the ratio H_2CO_3: $NaHCO_3$ for the arterial blood and C is that for the *OC* line, giving the normal C_H.) The evidence already available indicates that all four conditions occur in man.

If names are wanted for these four conditions we would suggest hypercapnia (17), excess of carbonate, for every sort of condition in which the $NaHCO_3$ in the blood is unusually high, and hypocapnia for all in which it is low; acidosis when $A > C$ and C_H is above normal, and alkalosis when $A < C$ and C_H is below normal.

These statements, particularly in relation to decompensation to the right or acid side of the diagram, are illustrated in Figs. 5, 6, and 7. Fig. 5 and Experiments 10, 11, and 12 show that, when the rate or amount of acid introduced into the blood exceeds

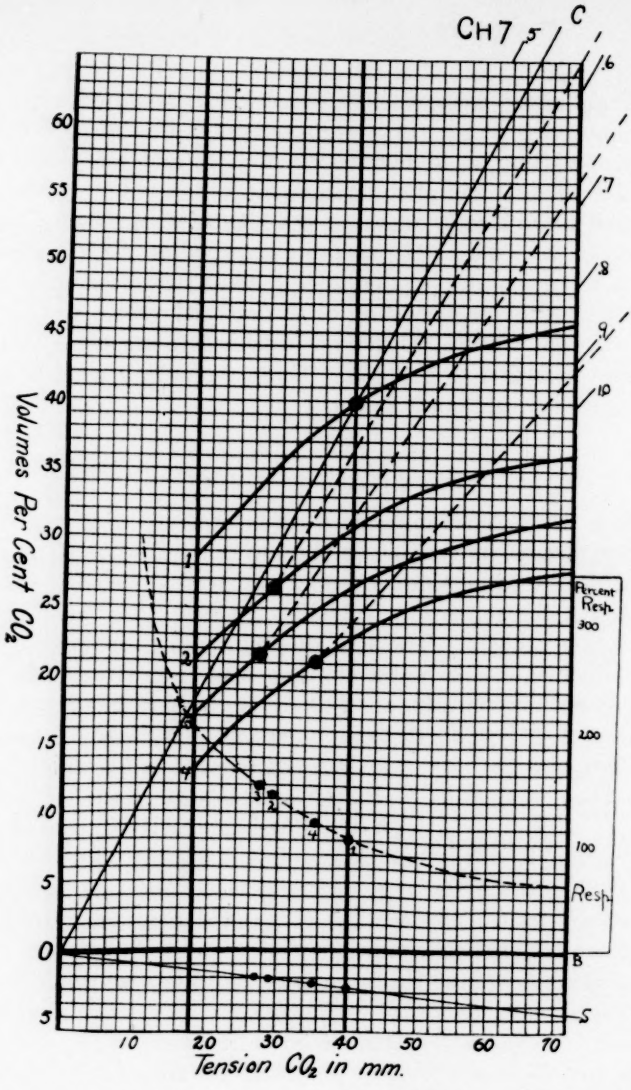

FIG. 5. The CO_2 diagram during progressive and finally fatal acidosis. From the data of Experiment 11.

The volume of breathing needed to compensate a lowered alkali is that point on the curve Resp. vertically below or above the intersection of the *OC* line and the dissociation curve. Actually the dots 2, 3, and 4 on curve Resp. fall to the right of these positions and indicate incomplete respiratory compensation. Dissociation Curve 4 would meet the *OC* line so near *O* as to involve a wholly impossible volume of breathing. Death inevitably follows.

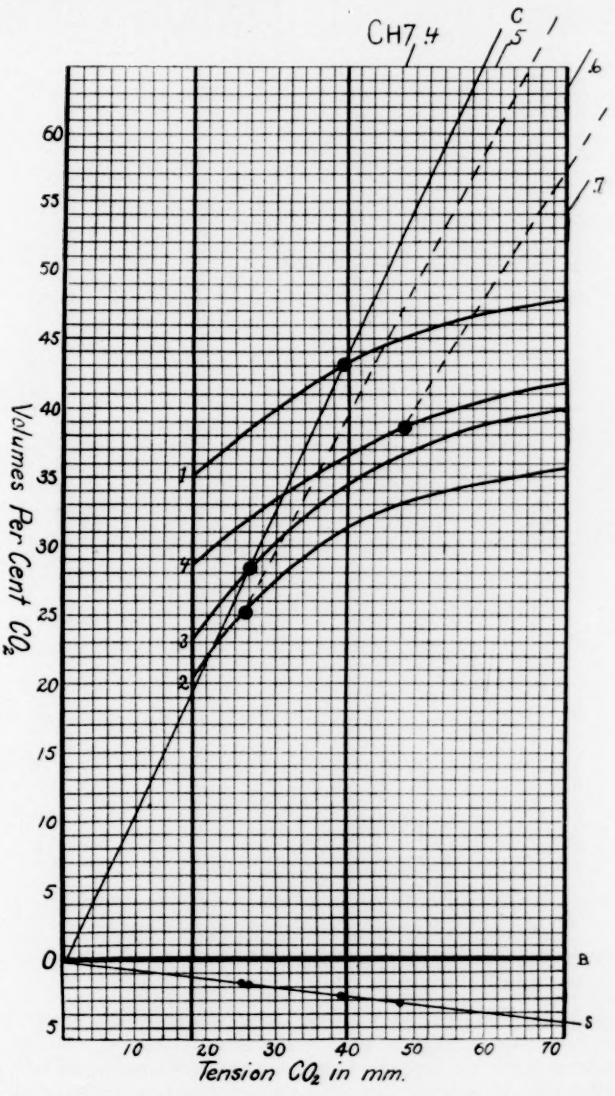

FIG. 6. The CO_2 diagram of Experiment 14, showing that inhalation of CO_2 in amounts harmless to a normal animal causes death after administration of acid.

Curve 1, normal; 2, after injection of acid; 3, during recovery; and 4, during inhalation of CO_2 with a rise of C_H7 to 0.655 (0.67 in the figure, see Experiment 14),—and death.

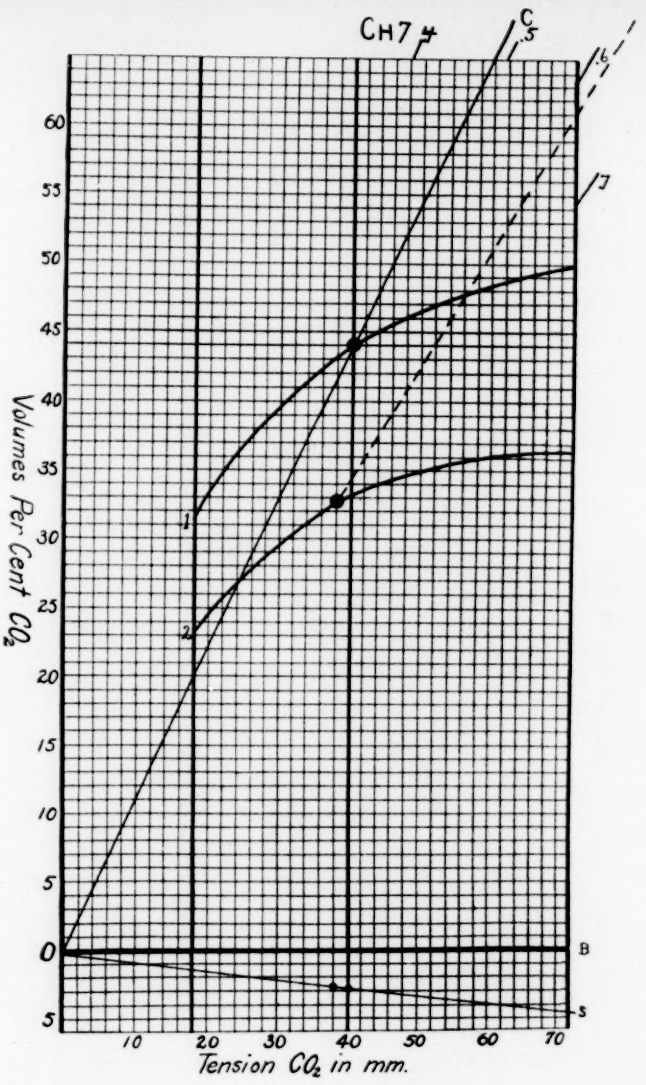

FIG. 7. The CO_2 diagram of Experiment 16, in which the animal was made to rebreathe through a dead space of only 200 cc.,—harmless to a normal animal. It is here seen that although the amount of HCl administered was such as without the rebreathing would easily have been compensated, the extra load on respiration prevents compensation and death results. The comparatively low fatal C_H in this and the preceding experiment is possibly to be explained by the extreme exertion of the respiratory muscles.

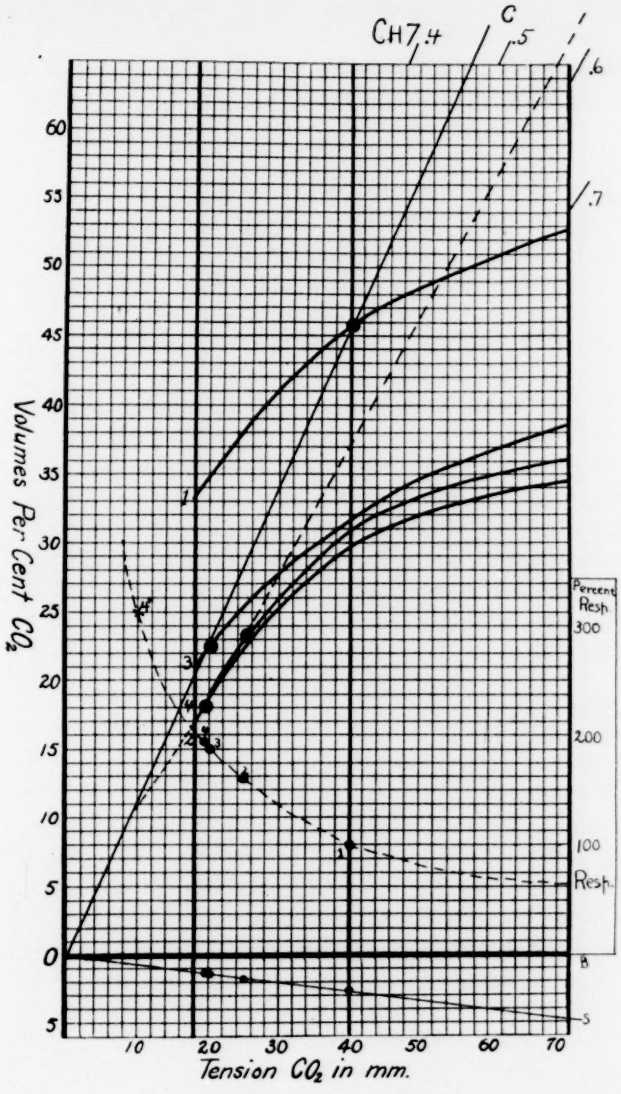

FIG. 8. The CO_2 diagram of Experiment 17, showing the effect of morphine after acid administration. The depression of respiration prevents compensation and death results. Dot 4 on curve Resp. shows the actual breathing; $x4'$ shows the volume needed for compensation. (Slight inaccuracies in some analyses and in the plotting of the data in the figure occur here (see Experiment 17), but do not invalidate the diagram.)

the capacity of the organism, respiratory compensation fails. Not only is the dissociation curve lowered, but the arterial point moves far to the right. C_H rises correspondingly, and death results.

Figs. 6 and 7 illustrate experiments (Nos. 13–16) in which a moderate dosage of acid was employed, but was reenforced either by slight rebreathing of expired air or by inhalation of 5 to 6 per cent CO_2. Normal animals would easily have sustained this load upon respiration, but these animals were killed by it at an increase of C_H equivalent to that of the fatalities in the preceding experiments with acid alone.

In Fig. 8 (from Experiment 17) the administration of morphine after acid injection is seen to have caused death in essentially the same way; namely, by respiratory decompensation.

These observations illustrate how essential is the part which increased volume of breathing plays in compensating hypocapnia (low dissociation curve) and thus preventing acidosis. In the CO_2 diagrams of some of the fatal cases it will be noted that the dissociation curve fell to a level such that the curve would meet the *OC* line at or near zero. No possible effort of respiration could bring the dissolved CO_2: combined CO_2 ratio to a value affording a normal C_H.

The Meaning of Decompensation.

Experiments 10 to 17 present in a very clear form the problem of respiratory decompensation, or what is now called "uncompensated acidosis" (18). If C_H is the normal hormone of respiration and the arterial CO_2 values normally fall on the intersection of the dissociation curve and the *OC* line, as Experiments 1 to 9 clearly demonstrate, why is there the striking deviation from this condition which is to be seen in Experiments 10, 11, and 12 when the administration of acid was more rapid?

The deviation from the *OC* line cannot, we think, be due to a merely mechanical inability of respiration to keep up with the mass of CO_2 to be eliminated. The augmentation of breathing in such experiments falls short of that degree of activity which in a normal animal can be called forth by muscular work, by inhalation of CO_2, or by deficiency of oxygen.

The breathing in extreme respiratory decompensation is of a peculiar gasping type. Inspiration and expiration do not follow each other with that smoothness which in normal respiration and especially in the hyperpnea of physical work reminds one of a smoothly running reciprocating engine with a heavy fly-wheel. In acidosis on the contrary the respiratory mechanism appears, as it were, dead centered, or jammed, or at least lacking in momentum.

These observations on the relative inadequacy of respiration in acidosis in our experiments are in accord, we believe, with those of Hooker, Wilson, and Connett (19), although their interpretation was quite different. They perfused the medulla oblongata alternately with blood rich in CO_2 and with blood of the same C_H but partly neutralized by HCl and therefore of less CO_2 content and tension. They found that the effect of the former in augmenting respiration was much the more marked. Scott (20) has obtained a similar result in comparing the effects of CO_2 inhalation and acid injection in decerebrated cats.

Similarly Peters (21) has shown on man that the ratio of the alveolar CO_2 to the alkali of the plasma is increased in conditions of "uncompensated acidosis." (But in cases of cardiac decompensation and slowed circulation the difference in the amount of alkali in use in the venous and arterial blood may be enormous, and the ratio of alveolar CO_2 to the alkali of plasma centrifugalized from venous blood correspondingly misleading.)

Hooker, Wilson, and Connett think that their results indicate that the concentration of HCO_3 ions rather than that of H ions is the hormone of respiration, but the relation of the curve Resp. to the *OC* and *OS* lines in our diagram negatives this idea; for the one decreases as the other two increase.

Alteration of the sensitiveness of the respiratory center naturally suggests itself. This hypothesis is however scarcely susceptible of direct test. To prove it would require the exclusion of every other conceivable cause.

What the underlying nature of the phenomenon may be is not known. It is worth noting however that an alteration of the dissociation characteristic of the blood could play a part in such a condition. The possible influences of alterations of alkali in use upon the oxyhemoglobin dissociation curve and thus upon

the oxygen supply and tension of the respiratory center also require investigation. Alteration of the conditions underlying the shape of the CO_2 dissociation curve likewise would have to be taken into consideration.

In particular we may here mention a relation to be developed in a later paper in connection with a discussion of the third and fourth laws of respiration: There appears to be a normal ratio of oxygen tension to blood alkali. At a barometric pressure of 760 mm. it may be written $T_{O_2} : Q_{CO_2} :: 100 : 40$. Whenever the tension of oxygen falls below this ratio, or the alkali exceeds it, respiration tends to be stimulated beyond the requirements of the H_2CO_3: $NaHCO_3$ relation, and deviation of the arterial values to the left of the *OC* line and lowered C_H tend to occur (see Curve 2 in Fig. 4). On the other hand when the ratio of oxygen to alkali is greater than this proportion respiration tends to be depressed; *i.e.*, deviation to the right of the *OC* line occurs, and C_H rises above normal. Respiratory decompensation in acidosis is conceivably due to this cause: low alkali and normal oxygen, that is, a relatively high oxygen, just as in a normal person soon after descending from an altitude to which he has become acclimatized.

From a teleological standpoint it is clear from our previous work (14), formulated in the fifth law, that a condition in which the ratio H_2CO_3: $NaHCO_3$ is high, that is acidosis, is one which tends strongly to draw alkali into the blood. We shall show in later sections of this series that precisely the opposite condition occurs during adjustment to low oxygen. From our work as a whole it appears that the maintenance for a time of a high or low ratio of H_2CO_3: $NaHCO_3$ is the means by which the necessary increase or reduction of alkali is effected. It is the mechanism for restoring and holding the dissociation curve to its normal level.

Upon the main point, namely that in the interaction of H_2CO_3 (T_{CO_2}) and $NaHCO_3$ (Q_{CO_2}), C_H and not HCO_3 is normally the hormone of breathing, the evidence presented in this paper allows no doubt. This conclusion is confirmed by the fact that the evidence now available (FitzGerald (22), and (15), (16), (23), and (24)) indicates that normal men acclimatized to various altitudes from sea level to 14,000 feet would afford a series of dissociation curves, arterial points, and volumes of breathing essen-

tially similar in appearance to Fig. 2. The processes of adjustment to experimental acid injection and to low barometric pressure are as we shall show in the following paper in some respects diametrically the opposite of each other; the arterial point in the latter case descends at the left of the *OC* line. The similarity of the end conditions in the two cases indicates therefore the fundamental correctness of such a diagram as Fig. 2 as a picture of the normal acid: alkali equilibrium at all levels.

IV. The C_H7 Scale and the Dissociation Characteristic.

It will be noted that in the scale around the upper right hand corner of the CO_2 diagram the hydrogen ion concentration is expressed by the symbol C_H7. We find that the values expressed by the Sörensen (25) symbol, pH, are not convenient in this connection, while such an expression as $C_H = 0.4 \times 10^{-7}$ is unnecessarily elaborate. Instead we write $C_H7 = 0.4$. In terms of this expression the extreme values at all compatible with life for the arterial blood appear to lie between 0.3 and 1.0. To determine the C_H7 indicated by the *OC* line or any other line through *O* in the diagram use could be made of the values which have been worked out, especially by Michaelis and Rona (26) for the degree of dissociation of the various substances in the blood, carbonic acid, sodium bicarbonate, phosphates, and proteins, and for the powerful influence of hemoglobin.

It is simpler however to use merely the resultant of all these factors summarized in a single figure. It may be called the dissociation characteristic (Char.) of the blood. The formula which we use is as follows:

$$\frac{\text{Dissolved } CO_2}{\text{Combined } CO_2} = \frac{C_H7}{\text{Char.}} \quad \ldots\ldots (1)$$

or reducing the left hand side of this equation to a figure (R) expressing the ratio, dissolved CO_2: combined CO_2: : R (in per cent): 1.00, we have

$$R \times \text{Char.} = C_H7 \quad \ldots\ldots (2)$$

It will be seen that what we term the characteristic is virtually the same as K in L. J. Henderson's expression quoted above. On

TABLE I.

Showing for various tensions of CO_2 within vital limits the ratio of dissolved CO_2 to combined CO_2 (from the CO_2 tension and content) and the C_H of the blood found by previous investigators. From these data we have calculated the dissociation characteristic, (Char.)—within moderate limits of observational error, — a value = 8.

Source of blood.	Tension of CO_2 at 38° mm. mercury.	Dissolved CO_2 / Combined CO_2 = $\frac{R}{100-R}$	$C_H 7$.	Char.
Ox blood, defibrinated.	39.3	6.60	0.55	8.34
(Hasselbalch), *Biochem. Z.*, 1916,	28.7	5.35	0.446	8.35
lxxviii, 123.	63.1	9.71	0.728	7.50
	95.6	11.40	0.911	8.00
	10.7	2.27	0.191	6.80
	33.6	4.97	0.381	7.67
	20.1	3.51	0.269	8.00
	43.7	5.97	0.489	8.17
	61.2	7.54	0.616	8.17
	74.0	8.32	0.675	8.28
	96.7	11.0	0.857	7.71
Average				7.91
Normal human blood, defibrinated.	50.8	6.46	0.550	8.52
(Hasselbalch), *Biochem Z.*, 1916,	45.7	6.85	0.478	7.80
lxxviii, 123.	32.7	5.13	0.416	8.10
	18.5	2.66	0.282	7.74
	22.4	4.27	0.324	7.60
	80.7	9.70	0.758	7.37
Average				7.85
Blood from pregnant woman, de-	52.8	7.90	0.624	7.90
fibrinated.	35.7	6.01	0.478	7.95
	39.3	8.42	0.468	7.95
	56.8	7.59	0.603	7.93
	49.75	7.33	0.589	8.02
8 days after delivery.	37.4	5.74	0.457	7.95
(Hasselbalch), *Biochem. Z.*, 1916,	44.7	5.42	0.572	8.00
lxxviii, 136.	56.5	7.90	0.624	7.90
Average				7.95
Pernicious anemia, defibrinated.	27.4	3.47	0.288	8.3
(Hasselbalch), *Biochem. Z.*, 1917,	39.7	4.67	0.398	8.35
lxxxii, 284.	62.6	7.23	0.589	8.15
Average				8.27

TABLE I—*Continued.*

Source of blood.	Tension of CO_2 at 38° mm. mercury.	$\frac{\text{Dissolved } CO_2}{\text{Combined } CO_2} = \frac{R}{100}$	C_H7	Char.
Uremia, defibrinated.	21.8	2.68	0.295	8.0
(Hasselbalch), *Biochem. Z.*, 1917,	39.5	5.34	0.452	8.3
lxxxii, 284.	68.7	8.40	0.675	8.1
Average				8.1
After recovery.	29.5	4.20	0.328	7.85
	45.0	5.61	0.457	8.15
	58.9	6.90	0.567	8.2
Average				8.1
Arthritis Case 1.	33.3	5.14	0.416	8.1
Case 2. Both defibrinated (Hasselbalch).	37.7	5.39	0.431	8.1
Biochem. Z., 1917, lxxxii, 284.	63.7	7.55	0.624	8.25
Average				8.15
Ox blood, defibrinated.	43.5	7.66	0.59	7.7
	93.6	10.30	1.00	7.66
Plasma from this blood.	41.3	7.08	0.517	7.30
(Hasselbalch and Warburg), *Biochem. Z.*, 1918, lxxxvi, 416.	74.5	9.34	0.792	8.47
Blood partially centrifugalized.				
a. poor in corpuscles.	31.2	4.55	0.372	8.03
b. rich " "	31.2	5.96	0.452	7.6
(Hasselbalch), *Biochem. Z.*, 1916, lxxx, 254.				
5 per cent hemoglobin in 0.025 per cent $NaHCO_3$ solution.	94.2	12.72	0.977	7.6
	20.2	3.3	0.263	7.95
(Hasselbalch), *Biochem. Z.*, 1916, lxxviii, 130.	7.0	1.00	0.115	7.1

TABLE I—*Continued.*

Source of blood.	Tension of CO_2 at 38° mm. mercury.	$\frac{\text{Dissolved } CO_2}{\text{Combined } CO_2} = \frac{R}{100 - R}$	$C_H 7$.	Char.
Normal human blood, defibrinated and oxygenated.	36.9	5.68	0.479	8.43
	19.1	3.61	0.263	7.24
(Parsons), *J. Physiol.*, 1917, li, 448.	70.0	7.80	0.617	7.90
Average				7.86
Same blood deoxygenated with hydrogen.	37.4	5.40	0.389	7.21
	19.6	3.28	0.234	7.15
	72.1	7.49	0.569	7.60
Average				7.32
Same blood (deoxygenated) some months later after oxygen treatment.	29.3	5.17	0.346	6.70
	45.3	6.52	0.489	7.50
Average				7.20
Same blood + 0.04 per cent lactic acid.	31.3	5.68	0.446	7.86
	46.1	7.46	0.550	7.88
Average				7.87
Same blood + 0.05 per cent lactic acid.	25.6	5.63	0.389	6.92
	40.9	7.43	0.537	7.24
(Donegan and Parsons), *J. Physiol.*, 1919, lii, 318.				
Average				7.08
Gassed soldier (G.). Blood deoxygenated.	29.6	4.49	0.346	8.02
	44.0	5.56	0.457	8.22
Donegan and Parsons.	65.7	7.25	0.576	7.94
Average				8.06
Same blood (deoxygenated) after soldier had received oxygen treatment.	37.0	5.51	0.398	7.24
	67.0	8.34	0.645	7.70
Donegan and Parsons.				
Average				7.47
Gassed soldier (H.). Blood deoxygenated.	18.8	3.47	0.240	7.0
	31.5	4.38	0.363	8.28
	44.2	5.53	0.467	8.44
Average				7.91

TABLE I—*Concluded.*

Source of blood.	Tension of CO_2 at 38° mm. mercury.	$\frac{\text{Dissolved } CO_2}{\text{Combined } CO_2} = \frac{R}{100 - R}$	$C_H{}^7$.	Char.
After oxygen treatment. Blood deoxygenated.	18.1	3.05	0.240	7.86
	38.9	5.93	0.407	6.85
Donegan and Parsons.	59.3	7.37	0.563	7.64
Average				7.45
Gassed soldier (L.) after oxygen treatment. Blood deoxygenated.	20.0	2.76	0.263	7.00
	42.7	6.32	0.452	7.16
Donegan and Parsons.	65.9	8.30	0.588	7.08
Average				7.08
Gassed soldier (S.). Blood deoxygenated.	15.9	3.01	0.224	7.44
	38.2	5.60	0.398	7.10
	51.5	6.79	0.500	7.36
Average				7.30
After oxygen treatment. Blood deoxygenated.	21.3	3.68	0.279	7.60
	40.5	5.78	0.446	7.70
Donegan and Parsons.	60.0	7.70	0.563	7.73
Average				7.68
Gassed soldier (T.). Blood deoxygenated.	30.8	5.29	0.381	7.21
	45.9	6.84	0.467	6.88
Average				7.05
After oxygen treatment. Blood deoxygenated.	19.1	3.83	0.252	6.58
	40.8	6.41	0.416	6.50
Donegan and Parsons.	60.7	7.40	0.550	7.43
Average				6.84

It is possible that slight sources of error are involved in some of the data quoted in Table I: (1) The Barcroft-Haldane (28) method of determining the CO_2 content of blood used for some of these data is apt to give figures a little too low. We find that it is exceedingly difficult, if not impossible, to shake all the CO_2 out of a precipitate of blood, potassium ferricyanide, and tartaric acid; (2) Defibrinated blood was used for most of the observations quoted in this table, and such blood we find is liable to show a CO_2 capacity a little less than the original blood. This is due, in our experience, in part at least to a slight amount of laking. We find that liberated hemoglobin even in small amounts lowers the CO_2 capacity by several volumes per cent; (3) Parsons (27) has shown that deoxygenation lowers the C_H of blood, which would seem to us to involve a corresponding lessening of the characteristic. The point needs investigation on blood which has not been defibrinated.

theoretical grounds he took the value of K as 3.8. Michaelis and Rona, expressing the dissociation of H_2CO_3 and $NaHCO_3$ separately found 4.4×10^{-7} and 0.6×10^{-7}, from which we figure $\frac{4.4 \times 10^{-7}}{0.6 \times 10^{-7}} = 7.33$ Char.

Actually when one uses such measurements of the blood as those published by Hasselbalch and his collaborators and by Parsons (27), and Donegan and Parsons (29), the dissociation characteristic of the (arterialized) blood of animals and of men and women in health and disease shows remarkably slight variation from a mean value of 8.

We have summarized practically all the data available in Table I.

The rule which we use for convenience in calculating and placing the C_H7 scale around the upper right hand corner of the CO_2 diagram is as follows: Having obtained the characteristic, and (arbitrarily) taking the tension of CO_2 as 42 mm. and the dissolved CO_2 therefore as 2.82, the ordinate of the line OS at 42 mm., we calculate the position of the line representing a C_H7 of 1.0 as follows:

$$\text{Combined } CO_2 = \frac{\text{Char.} \times \text{dissolved } CO_2}{C_H7} = \frac{8 \times 2.82}{1.0} = 22.6$$

volumes per cent CO_2.

This value, 22.6 volumes per cent combined CO_2, is now measured upward from OB on the vertical line in the diagram corresponding to 42 mm. CO_2. At the height found a point is made and a ruler is laid from this point to 0, the origin of coordinates and the line marked $C_H7 = 1.0$ is drawn. The positions of the other values of C_H7 are obtained by placing them in the equation in place of 1.0.

A slight increase or decrease of the characteristic would shift the C_H7 scale on the CO_2 diagram to the left or right respectively.

The final proof of the validity of the CO_2 diagram and of the OC line as an expression of normality is afforded by the fact that in every set of observations quoted in Table I the relations of T_{CO_2}, Q_{CO_2}, and C_H conform to the requirements of the diagram.

CONCLUSIONS.

1. The CO_2 diagram of the blood here developed shows the relations of the tension and content of CO_2, the alkali in use, and the C_H.

2. Under intravenous administration of acid (HCl) the dissociation curve of the blood is progressively lowered, but the arterial values follow down, or after brief deviation they return to, the line of uniform ratio of H_2CO_3: $NaHCO_3$,—that is uniform C_H, the so called *OC* line. This compensation is effected by respiration, and is maintained as long as the amount and rate of acid injection do not considerably exceed the capacity for increased breathing. These points demonstrate that C_H, and not HCO_3, is normally the hormone of respiration.

3. Under excess of acid respiratory compensation fails, a deviation of the arterial values to the right or acid side of the *OC* line occurs, and C_H rises until death results.

4. During a condition even of slight acidosis inhalations of CO_2 or rebreathing, in amounts otherwise harmless, cause a further deviation to the acid side by preventing compensation, and are fatal.

5. Morphine in true acidosis is likewise peculiarly toxic.

6. The administration of alkali on the contrary raises the dissociation curve. It may cause a temporary deviation of the arterial values to the left or alkaline side of the *OC* line, but the arterial point returns to the intersection of the dissociation curve and the *OC* line, which it then descends.

7. From the CO_2 diagram it is evident that it is necessary to distinguish clearly between (*a*) an elevation or lowering of the dissociation curve, that is the amount of usable blood alkali, and (*b*) a deviation of the arterial values to the right or left of the *OC* line, in other words high or low C_H. The latter conditions are determined by an inadequate or an excessive pulmonary ventilation.

8. The CO_2 diagram shows us that instead of only two forms of deviation from normality, acidosis and alkalosis, there are theoretically four possible abnormal conditions with all gradations between them: (1) high $NaHCO_3$ and $A > C$, high C_H; (2) low $NaHCO_3$ and $A > C$, high C_H; (3) high $NaHCO_3$ and $A < C$,

low C_H; and (4) low $NaHCO_3$ and $A < C$, low C_H. (A is the ratio H_2CO_3: $NaHCO_3$ for the arterial blood and C is that for the OC line, giving the normal C_H.) The evidence already available indicates that all four conditions occur in man. It is here pointed out that on the basis merely of measurements of blood alkali conditions which are essentially antithetic are now commonly regarded as identical.

9. Five laws of the hemato-respiratory functions are here formulated. The first is a modified form of that of Haldane and Priestley. The second, which is here proved, is the experimental confirmation of the formula derived from theoretical considerations by L. J. Henderson. The third and fourth, here stated only in provisional form, apply to the influence of oxygen or its deficiency upon breathing and to respiratory decompensation, and will be discussed further in later papers. The fifth is based on our previous work, and indicates the part which respiration plays in maintaining, or regaining, the amount of blood alkali normal for the barometer.

10. The data of previous investigators are here assembled to show that the dissociation characteristic of the arterial blood has a value approximating 8.

Experiment 1.—Dog, male, 10 kilos. Data for normal CO_2 dissociation curve, with values for arterial and venous blood. The ratio of dissolved CO_2 to combined CO_2 is expressed under R.

The data of this experiment are expressed graphically in Fig. 1, and are discussed in its legend.

	Blood equilibrated with CO_2 at			CO_2 content.	CO_2 dissolved.	CO_2 combined.	R	C_H7 (Char. 8).	C_H7 (Char. 7).
	18 mm.	40 mm.	72 mm.						
	Volumes per cent.			Volumes per cent.					
							per cent		
Data for curve.....	32	45	53						
Arterial.....				46	2.75	43.25	6.36	0.520	0.445
Venous.....				50	3.69	46.31	7.9	0.632	0.508

Experiment 2.—Dog, male, 11 kilos. Administration of acid with nearly complete compensation. Normal HCl administered intravenously at the rate of 0.25 cc. per minute for 3 hours. Data plotted in Fig. 2.

Time.	Total acid administered.	Blood equilibrated with CO_2 at			Arterial blood.			R	C_H7 (Char. 8).
		18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.		
		Volumes per cent.			Volumes per cent.				
	cc.							per cent	
11.30	0	34	48	57	50	3.03	46.97	6.45	0.516
1.30	22.5	28	40	48	37	2.26	34.74	6.51	0.521
3.00	45	21	33	40	24	1.49	22.51	6.52	0.530
5.30		25	37	47	31	1.90	29.10	6.54	0.523

Experiment 3.—Dog, male, 11 kilos. Normal HCl administered intravenously at the rate of 0.2 cc. per minute. Nearly perfect compensation and tendency to recovery.

Time.	Total acid administered.	Blood equilibrated with CO_2 at			Arterial blood.			R	C_H7 (Char. 8).
		18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.		
		Volumes per cent.			Volumes per cent.				
	cc.							per cent	
12.00		28	42	55	43	2.82	40.18	6.99	0.559
1.30	18	24	37	48	32	2.08	29.92	6.95	0.556
3.00	36	23	30	39	26	1.74	24.26	7.17	0.573
4.30		25	36	49	31	2.01	28.99	6.93	0.554

Experiment 4.—Dog, male, 14 kilos. Normal HCl administered intravenously 0.2 cc. per minute. Slight decompensation. Tendency to recovery.

Time.	Total acid administered.	Blood equilibrated with CO_2 at			Arterial blood.			R	C_H7 (Char. 8).
		18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.		
		Volumes per cent.			Volumes per cent.				
	cc.							per cent	
12.00	Normal.	32	43	52	45	2.9	42.1	6.88	0.551
1.40	19	29	39	47	37	2.37	34.63	6.86	0.549
3.00	36	25	34	40	31	2.08	28.92	7.23	0.574
4.00		26	38	45	34	2.21	31.79	6.95	0.556

Experiment 5.—Dog, female, 13.4 kilos. Normal HCl administered 0.3 cc. per minute. Slight decompensation.

Time.	Total acid administered.	Blood equilibrated with CO_2 at			Arterial blood.			R	C_H7 (Char. 8).
		18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.		
		Volumes per cent.			Volumes per cent.				
	cc.							*per cent*	
12.00	Normal.	35	49	57	50	2.95	47.05	6.26	0.501
1.00	18	28	39	46	35	2.07	32.93	6.29	0.503
2.00	35	24	35	40	28	1.74	26.26	6.63	0.530

Experiment 6.—Dog, male, 18 kilos. 44 cc. of normal HCl administered intravenously in 10 minutes. A considerable degree of recovery within 3 hours. Data plotted in Fig. 3.

Time.	Total acid administered.	Blood equilibrated with CO_2 at			Arterial and venous blood.			R	C_H7 (Char. 8).
		18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.		
		Volumes per cent.			Volumes per cent.				
	cc.							*per cent*	
11.40		32	47	54	Arterial. 48	2.87	45.13	6.20	0.497
					Venous. 55	4.98	50.02	7.89	0.790
12.00	44								
12.20		17	29	36*	Arterial. 22	1.68	20.32	8.25	0.660
					Venous. 37	4.90	32.10	14.80	1.184
3.00		24	40	51	Arterial. 34	2.10	31.90	6.43	0.510
					Venous. 43	3.18	39.82	7.97	0.630

* This figure is probably a little too low (due to incomplete gas equilibration or analytical error), and the right hand part of Curve 2 in Fig. 3 is correspondingly too low.

Experiment 7.—Dog, male, 11 kilos. Normal HCl, 0.5 cc. per minute. Temporary decompensation and complete recovery.

Time.	Total acid administered.	Blood equilibrated with CO_2 at			Arterial blood.			R	C_H7 (Char. 8).
		18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.		
		Volumes per cent.			Volumes per cent.				
	cc.							*per cent*	
11.30	0	29	44	54	45	2.88	42.12	6.84	0.547
12.40	20	22	32	40	27	1.74	25.26	6.89	0.559
1.40	50	18	28	34	21	1.48	19.52	7.59	0.606
24 hrs. later.	0	30	46	53	45	2.55	42.45	6.01	0.481

Experiment 8.—Dog, male, 16 kilos. Administration of 25 cc. normal HCl and later 50 cc. of 2 per cent $NaHCO_3$.

Time.	Blood equilibrated with CO_2 at			Arterial blood.			R	C_H7 (Char. 8).
	18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.		
	Volumes per cent.			Volumes per cent.				
							per cent	
11.35	33	45	55	45	2.68	42.32	6.33	0.506
12.00–12.15 HCl								
12.30	27	37	44	32	1.88	30.12	6.24	0.499
12.31–12.40 $NaHCO_3$								
12.45	35	49	60	52	2.80	49.20	5.69	0.455

Experiment 9.—Dog, male, 16 kilos. Administration of 4 per cent $NaHCO_3$, 6 cc. per minute. Rapid compensation at a high level. Data plotted in Fig. 4.

Time.	Total alkali administered.	Blood equilibrated with CO_2 at			Arterial blood.			R	C_H7 (Char. 8).
		18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.		
		Volumes per cent.			Volumes per cent.				
	cc.							*per cent*	
11.55	0	29	43	53	43	2.68	40.32	6.65	0.532
12.16	96	44	57	67	62	3.55	58.45	6.07	0.486
12.46		41	55	63	60	3.76	56.24	6.68	0.534
1.45		35	47	58	50	3.10	46.90	6.61	0.529

Experiment 10.—Dog, male, 10 kilos. Administration of HCl, 0.6 cc. per minute. Decompensation. Acidosis. Death in convulsions.

Time.	Total acid administered.	Blood equilibrated with CO_2 at			Arterial blood.			R	C_H7 (Char. 8).
		18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.		
		Volumes per cent.			Volumes per cent.				
	cc.							*per cent*	
12.00		33	46	55	45	2.61	42.39	6.15	0.492
12.30	18	25	38	46	33	1.94	31.06	6.20	0.496
1.00	36	19	30	35	27	2.06	24.94	8.25	0.660
1.05	Death.								

Experiment 11.—Dog, male, 11 kilos. Administration of normal HCl, 1.0 cc. per minute. Progressive decompensation, acute acidosis, and death in convulsions. Data plotted in Fig. 5.

Time.	Total acid administered.	Blood equilibrated with CO_2 at			Arterial blood.			R	C_H7 (Char. 8).
		18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.		
		Volumes per cent.			Volumes per cent.				
	cc.							*per cent*	
12.00		29	42	50	42	2.68	39.32	6.81	0.545
12.20	20	22	33	40	28	1.95	26.05	7.49	0.599
12.30	30	18	29	36	23	1.87	21.13	8.85	0.708
12.38	35	14	25	32	23	2.35	20.65	11.3	0.904
12.41	Death.								

Experiment 12.—Dog, male, 12.5 kilos. Administration of 0.5 N HCl, 3.0 cc. per minute. Progressive decompensation, acidosis, and death in convulsions.

Time.	Total acid administered.	Blood equilibrated with CO_2 at			Arterial blood.			R	C_H7 (Char. 8).
		18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.		
		Volumes per cent.			Volumes per cent.				
	cc.							*per cent*	
11.50		37	47	53	46	2.55	43.45	5.86	0.469
12.10	30	25	35	41	29	1.74	27.26	6.38	0.510
12.15	45	21	32	37	25	1.74	23.26	7.50	0.600
12.30	50	12	24	29	18	1.88	16.12	11.65	0.932
12.32	Death.								

Experiment 13.—Dog, female, 15.4 kilos. Administration of normal HCl, 0.3 cc. per minute; and inhalation of 6 per cent CO_2.

Time.	Total acid administered.	Blood equilibrated with CO_2 at			Arterial blood.			R	C_H7 (Char. 8).
		18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.		
		Volumes per cent.			Volumes per cent.				
	cc.							*per cent*	
11.45	0	31	42	48	41	2.51	38.49	6.5	0.520
1.15	22	28	38	45	35	2.18	32.82	6.65	0.532
1.30	27	26	35	41	35	2.80	32.20	8.70	0.696
1.35	Death.								

Experiment 14.—Dog, female, 12 kilos. Administration of normal HCl, 0.33 cc. per minute for 2.5 hours, followed 45 minutes later by inhalation of 5 per cent CO_2. Data plotted in Fig. 6.

Time.	Total acid administered.	Blood equilibrated with CO_2 at			Arterial blood.			R	C_H7 (Char. 8).
		18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.		
		Volumes per cent.			Volumes per cent.				
	cc.							*per cent*	
11.30	0	36	46	52	45	2.54	42.46	6.00	0.481
2.30	50	21	34	40	26	1.68	24.32	6.90	0.551
3.10		24	37	44	30	1.74	28.26	6.15	0.492
3.15	CO_2								
3.20		30	39	45*	41	3.10	37.90	8.20	0.655
3.31	Death.								

* This figure is probably a little too low, hence the slight discrepancy between the arterial point on Curve 4 in Fig. 6 and the C_H7 (0.655) in the data here tabulated.

Experiment 15.—Dog, female, 13 kilos. Administration of normal HCl, 0.5 cc. per minute. From 11.30 on the animal breathed through a dead space of 150 cc.

Time.	Total acid administered.	Blood equilibrated with CO_2 at			Arterial blood.			R	C_H7 (Char. 8).
		18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.		
		Volumes per cent.			Volumes per cent.				
	cc.							*per cent*	
11.45	0	29	42	53	44	2.96	41.04	7.21	0.576
12.30	15	24	38	48	36	2.48	33.52	7.40	0.592
1.00	30	22	34	43	32	2.46	29.54	8.34	0.667
1.10	Death.								

Experiment 16.—Dog, male, 14 kilos. Administration of normal HCl, 1.0 cc. per minute. From 12.00 on the animal rebreathed through a dead space of 150 cc. Data plotted in Fig. 7.

Time.	Total acid administered.	Blood equilibrated with CO_2 at			Arterial blood.			R	C_H7 (Char. 8).
		18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.		
		Volumes per cent.			Volumes per cent.				
	cc.							*per cent*	
11.45	0	32	47	54	47	2.69	44.31	6.07	0.486
12.25	25	24	36	40	35	2.54	33.46	7.82	0.624
12.36	Death.								

Experiment 17.—Dog, male, 9 kilos. Administration of 60 cc. of normal HCl, 1.0 cc. per minute. ½ hour later 0.135 gm. of morphine sulfate. Data plotted in Fig. 8.

Time.	Total acid administered.	Blood equilibrated with CO_2 at			Arterial blood.			R	C_H7 (Char. 8).
		18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.		
		Volumes per cent.			Volumes per cent.				
	cc.							per cent	
11.50		34	48	57	48	2.68	45.32	5.91	0.473
1.00	60	18	32	39	19	1.33	17.67	7.52	0.602
1.30	Morphine.	22	34	43	24	1.34	22.66	5.92	0.474
2.10		18	33	40	23	1.68	21.32	7.93	0.634
2.31	Death.								

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